Effects of Intracerebroventricular Bombesin Administration on Local Cerebral Glucose Utilization in the Restrained and Unrestrained Rat

BY

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To my family

for understanding my frequent absence

over the years

Abstract

Intracerebroventricular (ICV) administration of bombesin (BN) induces a syndrome characterized by stereotypic locomotion and grooming, hyperactivity and sleep elimination, hyperglycemia and hypothermia, hyperhemodynamics, feeding inhibition, and gastrointestinal function changes. Mammalian BN-like peptides (MBNs), *e.g.* gastrin-releasing peptide (GRP), Neuromedin C (NMC), and Neuromedin B (NMB), have been detected in the central nervous system. Radio-labeled BN binds to specific sites in discrete cerebral regions. Two specific BN receptor subtypes (GRP receptor and NMB receptor) have been identified in numerous brain regions.

The quantitative 2-[^{14}C]deoxyglucose ([^{14}C]2DG) autoradiographic method was used to map local cerebral glucose utilization (LCGU) in the rat brain following ICV injection of BN (vehicle, BN 0.1μg, 0.5μg). At each dose, experiments were conducted in freely moving or restrained conditions to determine whether alterations in cerebral function were the result of BN central administration, or were the result of BN-induced motor stereotypy. The anteroventral thalamic nucleus (AV) ($p=0.029$), especially its ventrolateral portion (AVVL) ($p<0.0005$), exhibited increased rates of metabolism under both restraint conditions. The effect was treatment dependent without interaction of the restraint conditions. Of all the regions that were reported to have high densities of BN receptors, the internal granular layer of the olfactory bulb (IGr) ($p=0.028$), and the suprachiasmatic nucleus (SCh) ($p=0.003$) exhibited BN treatment effects. BN effects on LCGU were also observed in the median eminence (ME) ($p=0.011$). Restraint, however, decreased LCGU in the lateral dorsal thalamic nucleus, ventrolateral and dorsomedial parts (LDVL and LDDM) ($p=0.044$, $p=0.009$), and the lateral geniculate (LG) ($p=0.027$).

In sum, BN induced a marked and highly localized alteration in cerebral metabolism within parts of the anterior thalamus, which is the principle relay in the limbic circuitry. BN effects were also observed in IGr, Mi, SCh, and ME. Effects of restraint were found in LDVL, LDDM, and LG. It is suggested that increased LCGU in AV and AVVL may be the result of functional change in the limbic circuitry and the hypothalamus caused by BN receptor functional modification. In IGr, increased LCGU following BN administration is considered to be mainly the result the activation of NMB receptor, a subtype of BN receptors. In SCh, increased LCGU is believed to be caused both by BN effects on the thalamic, the hypothalamic, and the limbic functions and by activation of GRP receptor, another BN receptors subtype found in SCh. In ME, increased LCGU is suggested to be caused by BN effects on the hypothalamic functions, especially those related to the neuroendocrine functions. None of the alterations seen in these regions reflects the emission of stereotyped motor behaviors. Rather, they reflect a direct influence of BN central administration upon functioning of the cerebral regions influenced by BN administration. The restraint effects seen in LD, including LDDM and LDVL, are suggested to be the result of altered behavioral expression. The restraint effects seen in LG is suggested to be the result of reduced locomotion.

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TABLE OF CONTENTS

List of Tables **Page**

List of Figures Page

Chapter 1. Introduction

Bombesin (BN) is a tetradecapeptide isolated from skin methanol extracts of a European amphibian of the family Discoglossidae, *Bombina bombina* (Anastasi *et al*, 1971) (Table 1). Several mammalian BN-like peptides (MBNs), which have amino acid sequence homology with BN, have been detected in the mammalian central nervous system (CNS), as well as in the gastrointestinal tract (Moody *et al*, 1986; Hernanz, 1990). The amino acid sequence of these MBNs and their structural homology with BN are shown in Table 1.

Table 1. Structural homology of BN-like peptides

Peptide	Structural homology														
BN	pGlu	Gln	Arg	Leu	Gly	Asn	Gln	Trp	Ala	Val	Gly	His	Leu	Met	NH_2
GRP	Met	Tyr	Pro	Arg	-	-	His	-	-	-	-	-	-	-	NH_2
NMC					-	-	His	-	-	-	-	-	-	-	NH_2
NMB					-	-	Leu	-	-	Thr	-	-	Phe	-	NH_2

BN ---- bombesin;
GRP ---- gastrin-releasing peptide, GRP_{14-27}*;
NMC ---- gastrin-releasing peptide(18-27), GRP_{18-27};
NMB ---- neuromedin B
- Identical amino acid sequence with bombesin;
* Numbers refer to the amino acid position from the N-terminus.
(modified from Ladenheim *et al*, 1990).

Bombesin central effects

BN intracerebroventricular (ICV) injection produces a syndrome which includes stereotypic grooming and locomotion, reduced sleeping and resting (Kulkosky *et al*, 1982; Rasler, 1984), and inhibited feeding (McCoy and Avery, 1990). It has been suggested that this BN syndrome may be mediated by a common mechanism involving endogenous MBNs (Moody *et al*, 1986; Hernanz, 1990).

Being a complex fixed-action pattern, stereotypic grooming and locomotion represent an exterior manifestation of the cooperative

function of multiple CNS circuits (Kupfermann, 1991c). Its execution requires an appropriate releaser, accurate movement maintenance, and appropriate termination. The accompanying sensory activation, proliferation of neuronal activity, and motor output may go far beyond any original localization of excitation and/or inhibition. Thus, a simple initiating factor, perhaps the activation of BN receptors, could lead to the involvement of multiple CNS systems in releasing behavioral stereotypy.

Central BN administration also affects hemodynamics. ICV injection of BN is followed by increases in both blood epinephrine and norepinepherine, resulting in tachycardia, and elevated blood pressure (Carver-Moore *et al*, 1991; Okuma *et al*, 1991). Central BN administration induces neuroendocrine changes and hyperglycemia. Furthermore, BN induces hypothermia under conditions of cold ambient temperature (4°C) (Brown *et al*, 1979), food deprivation, or insulin-induced hypoglycemia (Avery and Calisher, 1982; Babcock and Barton, 1989; Babcock *et al*, 1991).

Localization of mammalian bombesin-like receptors in the CNS

Perikaryal BN-like peptides have been localized in numerous cerebral regions (Panula *et al*, 1982, 1984; Costello *et al*, 1991; Watts and Swanson, 1987; Wada *et al*, 1990; Battey and Wada, 1991). BN receptors also have a widespread distribution (Zarbin *et al*, 1985, Wada *et al*, 1991). *In vitro* autoradiography, using iodine 125 labeled tyrosine BN ([^{125}I-Tyr^{4}]BN) to mark BN receptors, revealed numerous regions showing high [^{125}I-Tyr^{4}]BN densities (Table 2) (Zarbin *et al*, 1985; Moody *et al*, 1988). Binding was competitive, specific, saturable and reversible (Moody *et al*, 1978). *In vitro* binding of iodine 125 labeled gastrin-releasing peptide (GRP) to rat brain membranes confirms the existence of a specific

receptor for GRP in the brain (Hollingsworth, 1989).

Table 2. [^{125}I-Tyr^{4}]BN binding sites in the CNS

High binding level:
olfactory bulb and tubercle, nucleus accumbens, suprachiasmatic
periventricular nuclei of the hypothalamus
central medial thalamic nucleus
medial amygdaloid nucleus, hippocampus, dentate gyrus, subiculum
nucleus of the solitary tract, substantia gelatinosa
Moderate binding level:
parietal cortex, deep layers of neocortex, entorhinal cortex
caudate putamen, stria terminalis, locus coeruleus
parabrachial nucleus, facial nucleus
Low binding level:
globus palidus, lateral thalamus, midbrain
Negligible binding level:
cerebellum, corpus callosum

(from Zarbin *et al*, 1985; Moody *et al*, 1988)

The suggestion of specific receptors for BN and MBNs is well supported. Recent cDNA cloning studies demonstrate the existence of both neuromedin-B-prefering and gastrin-releasing-peptide-prefering BN receptors (Wada *et al*, 1991; Battey and Wada, 1991). *In situ* hybridization further revealed the distinct regional expression of neuromedin B (NMB) receptor mRNA and GRP receptor mRNA in the rat brain (Table 3), indicating that these BN receptor subtypes may participate in distinct neural functions. Studies using BN antagonists have also suggested the existence of at least two receptor subtypes, and have characterized the kinetics of their binding with BN, NMB, and GRP (Jensen and Coy, 1991). Ligand displacement studies have revealed that the GRP receptor has a high binding affinity and relative low capacity, whereas the NMB receptor has a relatively high capacity but lower affinity (Narayan *et al*, 1990).

Table 3. Distributions of GRP, NMB, GRP receptor, and NMB receptor mRNAs in the CNS receptor mRNA

Tissue	GRP	NMB	GRP receptor	NMB receptor
Forebrain				
Isocortex				
II	+ +	-	+ +	-
III	+ +	-	+	-
V	+	-	-	+ +
VI	+	-	-	+
Olfactory regions				
Main Bulb	-	+ + +	-	-
Anterior olfactory n.	+ +	-	(+)	+ + +
Hippocampal formation				
Dentate gyrus	+ +	+ +	+ +	+ +
Amygdala				
Amygdalohippocampal area	+ +	-	(+)	+ + +
N. lateral olfactory tract	-	-	+ + +	(+)
Basal ganglia				
N. accumbens	-	+	+	-
Magnocellular n.	+	-	+ + +	+
Thalamus				
Paraventricular n.	-	-	-	+ + +
Central median. and central lateral n.	-	-	-	+ + +
Hypothalamus				
Supraschiasmatic n.	+ +	-	+ + +	-
Supraoptic n.	-	(+)	+ +	-
Paraventricular n.	+	-	+ +	(+)
Medial preoptic n.	+ +	-	+ + +	-
Lateral mammillary n.	-	-	+ + +	-
Brainstem				
N. solitary tract	+ +	-	+	+
N ambiguus	-	-	+ +	+ +
Central gray	+	-	+	+

The strength of hybridization signal is graded based on the grains per positive cell. +++ Strong signal; ++ moderate strong; + weak signal; (+) very weak signal; - no signal over the background. Abbreviation: n.= nucleus. (From Battey and Wada, 1991)

However, the studies mentioned above have a common limitation. They could only provide possible sites of initial interaction between BN and the CNS. They can not reveal BN effects at sites remote from BN perikaryon, or terminal fields. Considering that the CNS functions as an interconnected network of neural circuits, the complex integrated behaviors induced by BN probably involve many cerebral regions. A fuller description of BN effects in the CNS requires mapping of BN influences upon functional

activity over large areas of the CNS. The mapping may not only show the locations of pharmacologically relevant BN receptors, but also sites influenced by subsiquent to the activation of BN receptors.

Research strategy

There is a method which allows the mapping of regional functional activity following BN administration. The quantitative 2-[^{14}C]deoxyglucose ([^{14}C]2DG) autoradiographic method has been used extensively to examine changes in local cerebral glucose utilization (LCGU) resulting from many types of experimental treatments (Sokoloff, 1981; Sokoloff *et al*, 1989). Since glucose is the major energy source of neurons, glucose utilization can be considered to be a direct reflection of cellular energy consumption levels. Combining information on the distribution of MBN-containing neurons and BN receptors, with data from a metabolic study demonstrating functional effects of BN administration, we may be able to put together a more detailed picture of the sites of BN central actions.

Strategy for mapping local cerebral glucose utilization

The [^{14}C]2DG method maps functional activity in a large number of cerebral regions. Each region is an independent experiment. Thus, a large scale map of the brain is composed of many experiments. Given the large number of experiments, there is a high probability of type I error over entire body of data. Therefore, we would like to reduce the chance of type I error occurring among the areas of greatest interest, by placing this small number of regions into a distinct category.

The anterior thalamus is the principle synaptic relay connecting subcortical limbic structures with the limbic cortex (Kelly and Dodd, 1991; Kupfermann, 1991a). Preliminary studies in our laboratory have

indicated that the anteroventral thalamic nucleus, especially its ventrolateral portion, shows increased metabolism after ICV injection of BN (0.5 μg/5μl), while other regions that show high receptor binding densities were unaffected (Ramm, unpublished data). Therefore, we intended to pay particular attention to LCGU in the anterior thalamus, and we define its subdivisions as the primary structures of interest. The subdivisions of the anterior thalamus include: anterodorsal thalamic nucleus, anteromedial thalamic nucleus, anteroventral thalamic nucleus, and its ventral lateral portion (Table 4).

Table 4. Primary regions: subdivisions of the anterior thalamus

Cerebral Regions	Abbreviations
anterodorsal thalamic nucleus	AD
anteromedial thalamic nucleus	AM
anteroventral thalamic nucleus	AV
anteroventral thalamic nucleus, ventral lateral part	AVVL

We define secondary structures as those which exhibit high concentrations of BN receptors (Zarbin *et al*, 1985; Moody *et al*, 1988; Wada *et al*, 1991; Battey and Wada, 1991) (Table 5).

Table 5. Secondary regions: BN binding sites

Cerebral Regions	Abbreviations
internal granular layer of the olfactory bulb	IGr
mitral cell layer of the olfactory bulb	Mi
olfactory tubercle	Tu
deep layer of neocortex	NcDL
accumbens nucleus	Acb
basolateral amygdaloid nucleus anterior part	BLA
lateral amygdaloid nucleus	La
medial amygdaloid nucleus	MeA
posteromedial cortical amygdaloid nucleus	PMCo
dentate gyrus	DG
subiculum	S
stria termilalis nucleus	ST
mediodorsal thalamic nucleus	MD
periventricular hypothalamic nucleus	Pe
suprachiasmatic nucleus	SCh
locus coeruleus	LC
nucleus of the solitary tract	Sol
substantia gelatinosa	SubGel

Tertiary structures include limbic structures other than the anterior thalamus; regions immediately surrounding the anterior thalamus, and hypothalamic regions involved in the regulation of homeostasis. Three subcortical visual regions were also included since they have been reported to be affected by immobilization (Bryan Jr, 1983) (Table 6).

Table 6. Tertiary regions: limbic regions, thalamic regions, hypothalamic regions, subcortical visual pathways

Cerebral Regions	Abbreviations
cingulate cortex area 1	Cg1
cingulate cortex area 2	Cg2
cingulate cortex area 3	Cg3
hippocampus	Hi
entorhinal cortex	Ent
lateral mammillary nucleus	LM
medial mammillary nucleus	MM
laterodorsal thalamic nucleus, dorsal medial	LDDM
laterodorsal thalamic nucleus, ventral lateral	LDVL
ventral lateral thalamic nucleus	VL
ventromedial thalamic nucleus	VM
ventral posterolateral thalamic nucleus	VPL
ventral posteromedial thalamic nucleus	VPM
ventromedial hypothalamic nucleus	VMH
dorsal medial hypothalamic nucleus	DM
lateral hypothalamic area	LH
posterior hypothalamic area	PH
arcuate hypothalamic nucleus	Arc
infundibular stem	InfS
median eminence	ME
lateral geniculate	LG
oculomotor nucleus	*3*
superior colliculus	SC

By organizing the cerebral regions into three categories, we still face the possibility of encountering significant findings purely by chance. Using this categorization, however, we can decrease the number of regions tested within each category. Now that our primary interest regions only need four tests, the *p* values can be examined with greater confidence that they are not a statistical artifact.

To determine if BN affects overall brain metabolic rate, we also investigated whole brain weighted cerebral glucose utilization.

Effects of motor stereotypy on local cerebral glucose utilization

In examining the effects of BN on LCGU, there is the possibility that alterations in metabolism could result from the direct actions of BN on neural functions. Observed alterations in metabolism could also result from the motor stereotypy associated with BN administration. Furthermore, there is the possibility of an interaction effect, in which the BN treatment effect depends upon the restraint effect, or *vice versa* (Figure 1). Therefore, the experimental conditions needed to include both freely moving animals, and animals that were prevented from emitting motor stereotypy. A condition of complete physical restraint was thus used to allow examination of effects due to behavioral stereotypy, as well as those due to the interaction effects between BN treatment and the restraint conditions (unrestrained and restrained). By prohibiting the animals from performing locomotor and grooming behaviors, the restraint condition allows possible BN treatment effects to be distinguished from the effects of motor stereotypy.

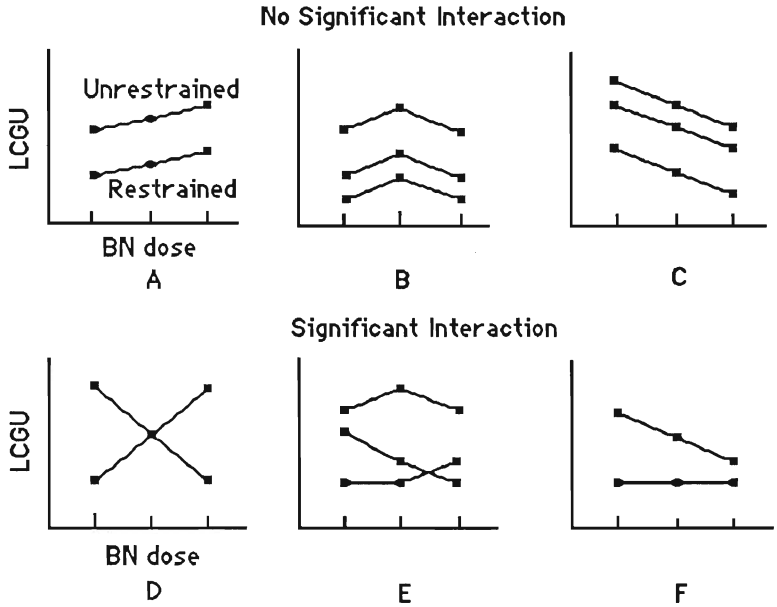

Figure 1. Example of possible scenarios of BN effects and restraint effects. The top three illustrate cases in which there is no interaction. The bottom three illustrate interaction effects. The rate of LCGU is not differentially affected by BN dose in restrained and unrestrained animals (Modified from Walsh, 1990; Daniel, 1991).

The classic [^{14}C]2DG method uses a pelvic plaster cast to immobilize the animal to facilitate the blood sampling procedures (Sokoloff, 1977). Increased stress caused by the pelvic plaster cast is an undesired side effect (Crane and Porrino, 1989). Our study requires complete restraint. We therefore designed a restraint jacket (Figure 3) using cloth material to "wrap up" the animal, and establish a reliable yet minimally stressful restraint condition. This jacket was considered to be more comfortable for the animal than other forms of restraint (plastic restrainer, or the plaster cast).

In sum, central BN administration causes a pharmacological syndrome characterized by stereotypic grooming and locomotion, hyperglycemia, hypothermia, hyperhemodynamics, neuroendocrine effects, and feeding inhibition. These behavioral and physiological changes may be mediated by BN receptors distributed in numerous brain regions. The [^{14}C]2DG method can be used to map regional cerebral metabolism following central BN administration. The objectives of the experiment were: 1) to investigate cerebral metabolism in the regions that have been linked to BN-induced behavioral stereotypy; 2) to investigate correlations between BN receptor distribution and regional cerebral metabolic alterations; 3) to investigate whether other cerebral regions were functionally involved in the BN-induced effects. In order to distinguish effects caused by BN receptor activation from those caused by motor stereotypy, animals were run under both restrained and unrestrained conditions.

Chapter 2. Literature Review:
The mediation of BN central effects

Following central BN administration, BN interacts with its receptors in the CNS. The effects of the interactions are twofold. Initially, the functional state of the cells endowed with BN receptors is modified. However, the scope of behavioral and physiological changes involved in the BN syndrome suggests involvement of many brain regions. Therefore, it is likely that there is synaptic transmission of the initial BN effects on BN receptors to other brain regions, where BN receptors may be absent.

Behaviorally, the BN syndrome involves excessive stereotypic grooming and locomotion (Kulkosky *et al*, 1982; Rasler 1984). At the physiological level, increased blood glucose levels (Brown *et al*, 1979), lowered body temperature set point (Stump *et al*, 1990; Avery *et al*, 1981; Babcock *et al*, 1989), agitated hemodynamics (Freeman *et al*, 1991), and altered enterogastric activities (Schubert *et al*, 1991; Strephens *et al*, 1991; Heymann-Mönnikes *et al*, 1990) are observed. The BN syndrome also includes feeding inhibition (McCoy and Avery, 1990).

In this chapter, the literature on BN central effects will be reviewed in the light of BN receptor mapping studies. We will also discuss possible functional connections between regions containing BN receptors and other brain regions which may be involved in mediating BN effects. At the end of the chapter, some of the theoretical concerns of the [^{14}C]2DG method will be examined.

I. Mapping of mammalian BN-like peptides and BN receptors in the CNS

Radioimmunoassay and immunohistochemical analyses have shown a wide distribution of two mammalian BN-like peptides (MBNs), *i.e.* gastrin-releasing peptides (GRP) and neuromedin B (NMB), in the rat brain (Panula *et al*, 1982, 1984; Moody *et al*, 1986; Watts and Swanson, 1987). *In situ* hybridization studies performed with cDNA probes specific for NMB or GRP mRNA in the brain have confirmed that GRP and NMB exist throughout the whole CNS (Wada *et al*, 1990). NMB mRNA is found most prominently in the olfactory bulb, dentate gyrus, and dorsal ganglion. In contrast, the highest levels of GRP mRNA are observed in the isocortex and hippocampal formation, including the hippocampus proper, dentate gyrus, and subiculum (Tables 3, 7) (Wada *et al*, 1990, Battey and Wada, 1991). The heterogeneous distribution of the various MBNs suggests that these structurally related neuropeptides have distinct functions.

Table 7. Distribution of strong cell expression of NMB mRNA and GRP mRNA

Cerebral regions	NMB mRNA	GRP mRNA
the olfactory bulb	+++	-
central thalamic regions	+++	-
dentate gyrus	+++	-
dorsal root ganglion	+++	-
isocortex	-	+++
hippocampal formation	-	+++

+++ Very high density of mRNA expression;
- No distribution.
(From Wada *et al*, 1991)

Just as BN-like peptides have been localized in numerous brain regions, BN receptors also have a widespread distribution (Zarbin *et al*, 1985). *In vitro* autoradiographic techniques to detect binding of [^{125}I-Tyr^{4}]BN to BN receptors have shown several regions that have high receptor densities (Moody *et al*, 1978; Wolf and Moody, 1985; Zarbin *et al*,

1985; Moody *et al*, 1988; Moody *et al*, 1990) (Table 2). Binding is specific, saturable and reversible (Moody *et al*, 1978). *In vitro* binding of iodine 125 labeled GRP ([^{125}I]GRP) to rat brain membranes has identified a high concentration of GRP receptors in the hippocampus, cortex, and striatum with high affinity (Hollingsworth, 1989).

Antibodies raised against BN block BN effects, indicating that BN effects are mediated by specific receptors (Merali *et al*, 1988b). Both central and peripheral administration of anti-BN antiserum have been shown to attenuate the effects of BN on feeding. Central anti-BN antiserum administration completely antagonizes the effect of BN on grooming (Merali *et al*, 1988b).

Ligand displacement studies have revealed functional variation between BN receptors. The GRP receptor, for example, has a high binding affinity and relatively low capacity (Narayan *et al*, 1990). In contrast the NMB receptor has a lower affinity and high capacity. Relative binding affinities of BN analogs are in the order of GRP > NMC ≥ BN ≥ $GRP_{1\text{-}27}$ > NMB (Narayan *et al*, 1990). Two BN receptor antagonists, [D-Arg^{1} D-Trp^{7,9} Leu^{11}]SP (spantide) and [Leu^{13} ψ(CH_2NH) Leu^{14}]BN (ψBN, the ψ symbol indicates substitution of a reduced peptide bond at the indicated site, *i.e.* CONH changes to CH_2NH) also inhibit specific binding of ^{125}I-GRP in a dose-dependent manner (Narayan *et al*, 1990). Functional variation of the receptors, as well as their structural homology may be responsible for the cross-sensitivity and limited specificity of the GRP receptors and NMB receptors.

[^{125}I-Tyr^{4}]BN and [^{125}I-Tyr^{4}]NMB binding studies have demonstrated that BN receptor heterogeneity exists within individual nuclei (Ladenheim *et al*, 1990). The nucleus accumbens exhibits high binding densities of

[^{125}I-Tyr^4]BN but not [^{125}I-Tyr^4]NMB. This binding is totally inhibited in the presence of BN, but only partially inhibited by NMB (Ladenheim *et al*, 1990), indicating that the predominant BN receptor in this region may be of the GRP type. The olivery nucleus shows similar receptor heterogeneity. In contrast to these different BN and NMB binding patterns, equivalent [^{125}I-Tyr^4]BN and [^{125}I-Tyr^4]NMB binding densities are observed in the nucleus of the solitary tract and paratrigeminal nucleus. This binding is inhibited in the presence of either BN or NMB (Ladenheim *et al*, 1990), indicating that the predominant BN receptor in this region may be of the NMB type. It has been suggested that the receptor heterogeneity found within various regions is associated with variation in regional response to central BN administration (Johnston and Merali, 1988 a,b).

Isolation of the GRP receptor and NMB receptor cDNA clones, from murine embryo fibroblasts and human lung carcinoma cells, provides the means to study BN receptor expression in the brain (Corjay *et al*, 1990, 1991; Battey *et al*, 1991; Wada *et al*, 1991). *In situ* hybridization studies of rat brain mRNA expression, using both NMB and GRP receptor probes, have shown the locations of BN receptor synthesis (Tables 3, 8) (Battey and Wada, 1991; Wada *et al*, 1991). Overall, NMB receptor mRNA expression is greatest in the olfactory and central thalamic regions, while GRP receptor mRNA expression is most prominent in the hypothalamus (Wada *et al*, 1991).

Table 8. Distribution of cell expression of NMB receptor mRNA and GRP receptor mRNA

Brain regions	NMB receptor mRNA	GRP receptor mRNA
tenia tecta	+ +	-
anterior olfactory nucleus	+ +	-
nucleus of the lateral olfactory tract	-	+ +
bed nucleus of the accessory olfactory tract	-	+
accessory olfactory bulb	+ +	-
piriform cortex	+ +	-
frontal cortex	+ +	-
isocortex	-	+
rhomboid	+ +	-
dentate gyrus	+ +	+
amygdalopiriform nucleus	+ +	-
lateral mammillary nucleus	-	+ +
field CA3 of Ammon's horn	-	+
medial amygdaloid nucleus	-	+
nucleus ambiguus	-	+
anterodorsal thalamic nucleus	+ +	-
central medial thalamic nucleus	+ +	-
central lateral thalamic nucleus	+ +	-
paraventricular nucleus	-	+ +
suprachiasmatic nucleus	-	+ +
magnocellular preoptic nucleus	-	+ +
lateral hypothalamic area	-	+
supraoptic area	-	+
dorsal raphe nucleus	+ +	-

++ High density mRNA expression;
+ Medium density mRNA expression;
- No expression.
(From Wada *et al*, 1991)

II. BN effects on animal behaviors

Intracerebroventricular (ICV) injection of BN induces behavioral stereotypy characterized by excessive grooming and overt locomotion (Kulkosky *et al*, 1982; Rasler, 1984). The animals appear to be trapped in a constantly activated state. Brief moments of sitting or lying always terminate with an abrupt burst of grooming or locomotor activity. The grooming appears compulsory (Rasler, 1984). Electroencephalographic analysis of BN injected rats reveals desynchronized or activated patterns consistent with the wakeful state (Rasler, 1984).

BN is not the only peptide that causes excessive stereotypic grooming and locomotion when given centrally (Cowan *et al*, 1985; Van Wimersama Greidanus, 1984; 1985 a, b; 1986; 1989). In rats, ICV injection of adrenocorticotropic hormone (ACTH) causes a dose-related behavioral stereotypy characterized by scratching (Cowan *et al*, 1985; Van Wimersama Greidanus, 1985a). ICV thyrotropin-releasing hormone (TRH) administration results in excessive grooming characterized by scratching and paw licking (Cowan *et al*, 1985; Van Wimersama Greidanus *et al*, 1989). In mice, ICV substance P (SP) administration (20 μg) induces both grooming and scratching behaviors for only 12 min, while BN (0.1 μg) induces enhanced grooming for about 2.5 hr (Meisenberg and Simmons, 1986).

In the rat, combined central administration of BN with TRH, or with a submaximal dose of ACTH does not result in more intense behavioral stereotypy than with single peptide treatment (Van Wimersama Greidanus *et al*, 1988). Tolerance (attenuated response beyound certain dose level) occurs for the effect of ACTH but not for that of BN. No cross-tolerance (attenuated response beyound certain dose level of other peptide) exits between BN and ACTH (Van Wimersama Greidanus *et al*, 1985), indicating that the behavioral effects of these peptides are mediated by their specific receptors (Gmerek and Cowan, 1983). However, due to certain similarities of the behavioral stereotypy, the mediation of the behavioral stereotypy induced by various peptides may share some common neural mechanism(s).

Locomotor and grooming behaviors are fixed-action patterns consisting of a combination of simpler fixed-action patterns performed in sequence (Kupfermann, 1991c). In rats, grooming behavior involves

movements of the head, directed toward the limbs, body, and tail, and movements of the fore- and hind-limbs, directed towards the head, body and tail. Grooming of the face is associated with two stereotypic motor patterns, including licking, single or parallel strokes with the forepaw. The various patterns do not occur in a random sequence, but rather in a relatively predictable order (Kupfermann, 1991c).

The distribution of BN receptors, and the effects of localized injection of BN indicate that numerous brain regions may be associated with initiating and/or maintaining BN-induced behavioral stereotypy, and with the physiological effects of BN. Since individual CNS regions are incorporated into complex neural circuits containing multiple brain regions (Kupfermann, 1991c), BN receptors in various cerebral regions may contribute to the behavioral and physiological effects of BN.

BN receptor distributions in limbic circuits

The limbic system, and the hypothalamic regions are characterized by widespread distributions of BN receptors in high concentrations (Tables 2, 3, 8) (Zarbin *et al*, 1985; Moody *et al*, 1988; Wada *et al*, 1991; Battey and Wada, 1991). In the limbic regions, GRP receptors are distributed in the dentate gyrus, fields CA3 of Ammon's horn, lateral mammillary nucleus, medial amygdaloid nucleus, and nucleus accumbens, whereas NMB receptors are distributed in the dentate gyrus, amygdalohippocampal area, and anterodorsal thalamic nucleus (Tables 3, 8) (Wada *et al*, 1991; Battey and Wada, 1991). The hypothalamic regions contain GRP receptors almost exclusively. These regions include: the suprachiasmatic nucleus, supraoptic nucleus, paraventricular nucleus, and medial preoptic nucleus (Tables 3, 8) (Wada *et al*, 1991; Battey and Wada, 1991).

The limbic lobe was first suggested to form a neural circuit that provides the anatomical substratum for emotions by James Papez in 1937 (Isaacson, 1974; McLean, 1976). In the limbic circuits, the anterior thalamus receives afferents from the cingulate cortex, hippocampal formation and mammillary complex of the hypothalamus (Figures 2, 18, 19). These various connections of the anterior thalamus complete circuits linking the hippocampal formation and hypothalamus with the cingulate (*i.e.* limbic) cortex (Kupfermann, 1991a; Faull and Mehler, 1985).

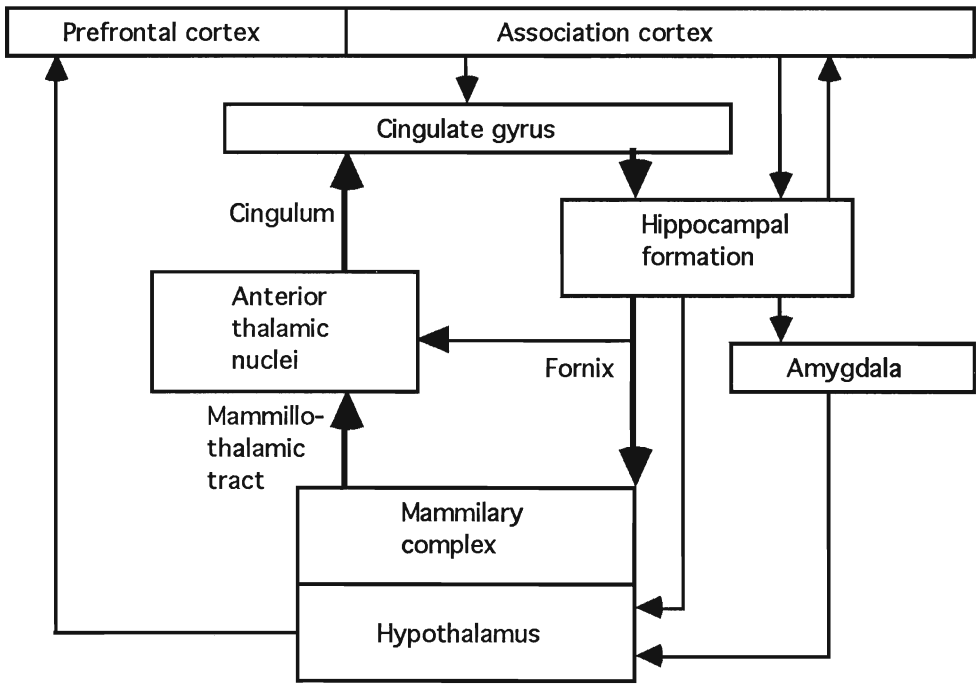

Figure 2. A proposed neural circuit for the limbic system. The circuit originally proposed by James Papez is indicated by thick lines; more recently described connections are shown by fine lines. Known projections of the fornix to hypothalamic regions (mammillary bodies and other hypothalamic areas) and of the hypothalamus to the prefrontal cortex are indicated. A pathway interconnecting the amygdala to limbic structures is shown. Reciprocal connections between the hippocampal formation includes the hippocampus proper and surrounding structures, including entorhinal cortex and the subicular complex (Modified from Kupfermann, 1991a).

The limbic system can be parceled into three main cortico-subcortical subdivisions (MacLean, 1986) (Figures 2, 18, 19). Two telencephalic nuclear groups located in the amygdala and the septum are centers for two of these subdivisions. *The amygdala division* has been

shown to be involved in feeling and expressive states essential for self-preservation (*i.e.*, feeding, fighting, and self-protection). *The septal division* has been found to be implicated in feeling and emotional expressive states. *The thalamocingulate division* is so referred to because the afferent supply to the mesocortical areas derives from the thalamus.

The laterodorsal thalamic nucleus, including the dorsomedial part and ventrolateral part, is a neothalamic nucleus linked to the limbic system. It lies just caudolateral to the anterior thalamus and peripheral to the internal medullary lamina (Faull and Mehler, 1985). The laterodorsal thalamic nucleus has reciprocal projections to the cingulate cortex (MacLean, 1986; Faull and Mehler, 1985). Its functions may be linked to emotional expression in mammals (Kelly and Dodd, 1991).

Afferent fibers to the anterior thalamus are contained in three major neural projections: the fornix, mammillothalamic tract (tract of Vicq-d'Azyr), and thalamocingulate fibers. Neural fibers from these projections usually go to specific anterior thalamic subdivisions (Figure 2) (MacLean, 1986): the anteroventral thalamic nucleus (AV), anteromedial thalamic nucleus (AM), and anterodorsal thalamic nucleus (AD). Hippocampal projections to the anterior thalamus arise from the subicular complex and pass via *the fornix* mainly to AV and AM (Faull and Mehler, 1985). The mammillary complex projects via *the mammillothalamic tract* to AV, AM and AD; in particular, the medial mammillary nucleus projects (bilaterally) to AD (Faull and Mehler, 1985). *The thalamocingulate tract* is largely composed of reciprocal fibers between the anterior thalamus and the cingulum. Afferents from the anterior thalamic regions project in a topographic fashion onto the medial (cingulate) cortex and also onto the presubiculum (Faull and Mehler, 1985). AV and AM also receive projections

from both the cingulate and retrosplenial cortices. (MacLean, 1986).

The anterior thalamus is composed of three cytologically distinct nuclei, AD, AV, and AM. These nuclei can be identified by their marked differences in acetylcholinesterase (AChE) staining intensity (Faull and Mehler, 1985; Paxinos and Watson, 1989). AV is especially prominent and well delineated in AChE stained material; it displays intense AChE activity, especially in its ventrolateral portion (AVVL). AD and AM are characterized by moderate and low levels of AChE activity, respectively. AM is composed of small to medium sized cells which stain less intensely than those in AD. AV is also characterized by its cellular density; the cells are closely packed, except in the caudal ventromedial region where they are often dispersed by fiber fascicles from the mammillothalamic tract (Faull and Mehler, 1985).

The mammillothalamic tract is a key neural pathway in the limbic circuitry. It has been reported that lesions of the mammillothalamic tract in the rat reduce retention in a two-way active avoidance task (Isaacson, 1974). Attenuated retention is believed to be the result of the animal's inability to perform the task due to transmission interruption. Amphetamine improves the active avoidance performance of normal animals as well as those with the mammillothalamic tract lesions (Isaacson, 1974), suggesting retention of the avoidance task can be improved by facilitation of behavior itself. The drug also enhances locomotor behavior and reduces "freezing" (Isaacson, 1974). Furthermore, the behaviors of the animals with mammillothalamic tract lesions (slow or reluctant to begin new behavioral acts) is similar to the behavioral changes caused by lesions of the limbic system which affect the animals' willingness to initiate or continue behavioral sequences (Isaacson, 1974).

Localized BN injection

Localized injections can deliver ligands (BN, MBNs, and BN antagonists) directly into regions containing BN receptors. There are several cerebral regions that are sensitive to BN administration. BN micro-injection into the hypothalamic areas, amygdala, periaqueductal gray, septum, ventral tegmantal areas, reticular formation (Kyrkouli *et al*, 1987), and nucleus accumbens (Moody *et al*, 1988) increases grooming behavior and decrease the time spent resting and sleeping.

However, due to the receptor heterogeneity between various brain regions and within individual nuclei, not all of the regions are equally sensitive. For example, the nucleus accumbens contains only GRP receptors, whereas the nucleus of the solitary tract contains both GRP and NMB receptors (Battey and Wada, 1991). It has been shown that BN (0.0001-1.0 μg) administrated into the nucleus accumbens did not significantly affect grooming or satiety, whereas both grooming and satiety were markedly increased by BN (0.0001-1.0 μg) micro-injection at the nucleus of the solitary tract (Johnston and Merali, 1988 a,b).

Monoaminergic system and BN effects

Central BN administration changes the functions of the tuberoinfundibular dopaminergic system (Manzanares *et al*, 1991). ICV BN has been demonstrated to cause a dose- and time-related increase in the activity of dopamine neurons projecting to the median eminence and intermediate lobe of the pituitary, and a corresponding increase in plasma growth hormone, and decreases in plasma prolactin and α-melanocyte-stimulating hormone (Houben and Denef, 1990). The stimulatory actions of GRP, NMC, and NMB on the anterior pituitary functions can be blocked by the BN

receptor antagonist ψBN (Houben and Denef, 1990). These effects are thought to be mediated through GRP receptors in the parvocellular neurons located in the paraventricular nuclei (Manzanares *et al*, 1991; Wada *et al*, 1991), and possibly those in the periventricular nuclei (Zarbin *et al*, 1985).

The mesolimbic dopaminergic system may also be affected by central BN administration. Although BN receptors have not been found in this system, voluntary movement coordination is associated with the function of the mesolimbic system (Role and Kelly, 1991). Since the BN-induced behavioral stereotypy involves accurate voluntary movement coordination, the mesolimbic dopaminergic system may play a role in the execution of the behavioral stereotypy induced by BN.

These suggestions are supported by several studies on the effects of BN on the mesolimbic dopaminergic system. Central BN administration increases the accumulation of dopamine metabolites in the striatum, olfactory tubercles, and hypothalamus, indicating increased dopamine turnover (Widerlov *et al*, 1984; Merali *et al*, 1985). When dopaminergic neurons are lesioned using 6-hydroxydopamine (6-OHDA, ICV), the behavioral effects of central BN are markedly attenuated or absent (Merali *et al*, 1985). Haloperidol [a dopamine receptor 1 (D_1) and dopamine receptor 2 (D_2) antagonist] has been shown to attenuate BN-induced behavioral stereotypy without changing the relative distribution of behavioral elements (Schultz *et al*, 1984; Van Wimersama Greidanus *et al*, 1985; 1986). However, BN-induced behavioral stereotypy is unaffected by behaviorally nondepressant doses of haloperidol, morphine, and neurotensin (Meisenberg and Simmons, 1986), indicating that the mesolimbic dopaminergic system has a facilitating function in modulating

BN-induced behavioral stereotypy.

Both D_1 and D_2 receptor based mechanisms participate in the modulation of BN behavioral effects (Merali and Piggins, 1990). The roles of D_1 and D_2 receptors differ in the modulation of BN-induced behaviors. D_1 is more involved with grooming and D_2 with locomotion, although both are required for the full expression of these behaviors (Piggins and Merali, 1989).

Serotonergic system. The raphe nuclei of the midbrain and upper pons project primarily in the medial forebrain bundle to an array of rostral sites, including the cerebral cortex, striatum, limbic structures, olfactory tubercle, hippocampus, and diencephalon (Role and Kelly, 1991).

The dorsal raphe nucleus expresses NMB receptor mRNA (Wada *et al*, 1991). Activation of BN receptors using BN, GRP, and NMB depolarize a subpopulation of dorsal raphe 5-HT neurons by acting on postsynaptically located receptors (Pinnock and Woodruff, 1990 a,b; Wada *et al*, 1991). Thus, the serotonergic system may also participate the modulation of BN induced behavioral stereotypy.

III. Bombesin effects on physiological functions

The effects of BN central administration on physiological functions reflect its effects on the hypothalamus. Both [^{125}I]BN binding studies and *in situ* cDNA hybridization maps of BN receptors indicate BN receptors in the suprachiasmatic nucleus, paraventricular nucleus, preoptic nucleus, supraoptic nucleus, lateral mammillary bodies, and periventricular nucleus (Zarbin, 1989; Wada et al, 1991; Battey and Wada, 1991). Therefore, BN effects on physiological functions may be mediated by hypothalamic BN receptors.

BN effects on neuroendocrine functions

Central BN administration affects the release of pituitary hormones. These activation are exerted via GRP receptors on the magnocelullar neurons in the paraventricular and supraoptic nuclei, and the parvocellular neurons in the paraventricular nucleus (Wada *et al*, 1991), and possibly the periventricular nucleus (Zarbin *et al*, 1985; Moody *et al*, 1988).

Functional modification of the paraventricular nuclei and supraoptic nuclei may cause increased vasopressin and oxytocin release from the magnocellular neurons (Armstrong, 1985; Kupfermann, 1991a), which is partially responsible for increased blood pressure following BN administration.

Functional modification of the paraventricular, and possibly periventricular nuclei causes the release of various inhibiting-releasing hormones from the parvocellular neurons. These inhibiting-releasing hormones are in turn transferred from the median eminence to the anterior pituitary through the pituitary portal system (Armstrong, 1985; Kupfermann, 1991a). Increases in plasma cortisone and ACTH concentration (Sander, 1991; Malendowicz *et al*, 1991), growth hormone (GH) (Bitar *et al*, 1991; Houben and Denef, 1990), and thyroid stimulating hormone (TSH) (Malendowicz and Miskowiak, 1990) are the results of functional modification in the hypothalamic nuclei following central BN administration.

BN effects on hemodynamics

ICV BN administration elicits a hemodynamic response similar to that of stress (Freeman *et al*, 1991). Increased blood epinephrine and norepinephrine are partially responsible for these reactions (Carver-Moore

et al, 1991; Okuma *et al*, 1991). Lateral ventricle injection only induces an increase in blood epinephrine, but not norepinephrine (Okuma *et al*, 1991).

Intracerebral microinfusion of BN into specific brain regions elicits increases in peripheral blood epinephrine and norepinephrine (Carver-Moore *et al*, 1991). An increase in both blood epinephrine and norepinephrine can be elicited by BN infusion into the central nucleus of amygdala, anterior hypothalamus, parabrachial nucleus, and nucleus of the solitary tract. Among these regions, the amygdala, and nucleus of solitary tract have high levels of BN receptor mRNA expression (Wada *et al*, 1991; Battey and Wada, 1991).

BN and circadian rhythm regulation

The suprachiasmatic nucleus has been identified as the primary biological clock which maintains CNS circadian rhythms (Kelly, 1991). Central BN administration affects a series of animal behaviors and physiological functions which have a circadian rhythm. [^{125}I-Tyr^{4}]BN binding studies support that there is high concentration of BN receptors in the suprachiasmatic nucleus. *In situ* hybridization studies confirm that the suprachiasmatic nucleus express GRP mRNA (Wada *et al*, 1990) and GRP receptor mRNA (Wada *et al*, 1991; Battey and Wada, 1991).

Immunohistological studies have confirmed that MBN-like pathways are sent out from the suprachiasmatic nucleus. These BN-like projections end in adjacent hypothalamic and thalamic regions (Watts and Swanson, 1987). Therefore, BN may affect circadian regulation through its effects on GRP receptors localized in the suprachiasmatic nucleus. BN may also affect circadian functions indirectly through its effects on other thalamic and hypothalamic regions.

BN effects on thermoregulation

ICV BN administration lowers body temperature when animals are kept under any of the following conditions: cold ambient temperature (4°C) (Brown *et al*, 1979); food deprivation; or insulin-induced hypoglycemia (Avery and Calisher, 1982; Babcock and Barton, 1989). BN is believed to lower the body temperature set point (Stump *et al*, 1990), which attenuates heat production and enhances heat loss. Two hypothalamic nuclei containing GRP receptors, the preoptic nucleus, and the paraventricular nucleus (Wada et al, 1991; Battey and Wada, 1991), may be responsible for mediating BN's hypothalamic effects.

BN microinfusion into the paraventricular nucleus has been shown to produce hypothermia if rats are kept either food deprived, or hypoglycemic with insulin. Refeeding in either of the conditions restores body temperature (Barton and Babcock, 1991; Babcock and Barton, 1990). BN microinfusion in the preoptic nucleus has similar effects (Babcock *et al*, 1992). BN's hypothermic effects in the preoptic nucleus have been shown to be antagonized with the BN antagonist ψBN (Babcock, *et al*, 1992), supporting the suggestion that BN hypothermic effects are mediated by BN receptors.

IV. BN central effects on feeding and digestive functions

Central BN administration specifically suppresses feeding at a dose that induces behavioral stereotypy (Kulkosky *et al*, 1982; McCoy and Avery, 1990; Meisenberg, 1990; Flynn, 1991). The feeding inhibition results from a reduction in the amount eaten, but not in the frequency of eating (Flynn, 1991). Bilateral injection of BN into the lateral hypothalamus, causes modification of post-deprivation feeding (Stuckey and Gibbs, 1982).

Micro-injection of BN produces strong suppression of feeding when injected into the hypothalamic areas, amygdala, and periaqueductal gray. The septum, ventral tegmantal areas, and reticular formation, however, are insensitive (Gibbs, 1985; Kyrkouli *et al*, 1987; McCoy and Avery, 1989). In all of these brain areas, BN microinjection increases behavioral stereotypy and decreases time spent resting and sleeping (Kyrkouli *et al*, 1987).

BN effects on gastric functions involve several systems. ICV BN administration inhibits gastric acid output and concentration (Strephens *et al*, 1991), and gastric contractility in rats (Heymann-Mönnikes *et al*, 1990). Central BN effects on gastric contractility can be antagonized by ICV injection of a BN antagonist [N-acetyl-GRP-(20-26)-OCH_3] (Heymann-Mönnikes *et al*, 1990), indicating the involvement of the autonomic neural mechanisms. Microinjected in the dorsal vagal complex, BN alone does not modify basal gastric contractility. However, BN inhibits the stimulation of gastric contractility induced by a thyrotropin-releasing hormone analog microinjected into the dorsal vagal complex (Heymann-Mönnikes *et al*, 1990), suggesting that BN participates in the modulation of gastric function by interacting with other ligand/receptor system.

The combined retrograde tracing and immunohistochemical technique, and localized BN receptor mRNA expression studies (Wada *et al*, 1991) provide evidence that links BN receptor functional modification with feeding inhibition (Costello *et al*, 1991). It has been demonstrated that the dorsal vagal complex receives direct projection from the parvocellular part of the paraventricular nucleus in the hypothalamus (Costello *et al*, 1991). In the paraventricular nucleus most of the vagally projecting BN immunoreactive neurons are located within the medial parvocellular

subdivision (Costello *et al*, 1991). BN-like immunoreactivity has also been localized within neuronal cell bodies of the hypothalamus and nerve terminals within the dorsal vagal complex (Panula *et al*, 1982, 1984). It has been hypothesized that a functional circuit relating these regions may mediate some of the feeding inhibition effects of central BN administration (Panula *et al*, 1982, 1984).

V. Theoretic basis of [^{14}C]2DG methodology

The [^{14}C]2DG method was used to map local metabolism levels in this study. The [^{14}C]deoxy-D-glucose ([^{14}C]2DG) method for the measurement of LCGU was derived by analysis of a model based on the biochemical properties of 2DG in central nervous tissue (Sokoloff *et al*, 1977; 1989). 2DG is transported bidirectionally between blood and brain by the same carrier that transports glucose across the blood-brain barrier. In the cerebral tissues, it is phosphorylated by hexokinase to 2-deoxyglucose-6-phosphate (DG-6-P). Deoxyglucose and glucose are competitive substrates for both blood-brain transport and hexokinase-catalyzed phosphorylation. Unlike glucose-6-phosphate, DG-6-P cannot be converted to fructose-6-phosphate, and it is also not a substrate for glucose-6-phosphate dehydrogenase. Other break down processes are slow, and the metabolites (DG-6-P) are relatively stable. The biochemical processing of 2DG is therefore stopped at this stage; DG-6-P is trapped in the cerebral tissue (Sokoloff *et al*, 1989).

If the DG-6-P accumulation time is kept short enough to allow the assumption of negligible loss of [^{14}C]DG-6-P from the neurons, then the quantity of [^{14}C]DG-6-P accumulated in cerebral tissue at any given time following the introduction of [^{14}C]2DG into the circulation is equal to the integral of the rate of [^{14}C]2DG phosphorylation by hexokinase in that

tissue during the accumulation time. This integral is in turn, related to the amount of glucose that has been phosphorylated over the same interval, depending on the time course of the relative concentrations of [^{14}C]2DG and glucose in the precursor pools and the Michaelis-Menten kinetic constants for hexokinase with respect to both [^{14}C]2DG and glucose. With glucose consumption in a steady state, the amount of glucose phosphorylation during the interval of time equals the steady-state flux of glucose through the hexokinase-catalyzed step times the duration of the interval, and the net rate of flux of glucose through this step equals the rate of glucose utilization (Sokoloff *et al*, 1989).

An operational equation (Appendix 2) is derived from the model, requiring the flowing assumptions:

1. Plasma glucose and the rate of cross-membrane glucose transportation remain constant throughout the experimental period;
2. Tissue compartment is homogeneous, within which the concentrations of [^{14}C]2DG and glucose are uniform and exchange directly with the plasma; and
3. Molecular concentrations of free [^{14}C]2DG are essentially equal to zero (Sokoloff *et al*, 1989).

Because the operational equation of the method was derived on the basis of the assumption that arterial plasma glucose concentration remains constant during the experimental period, the original method is applicable only to experiments in which this assumption is satisfied (Savaki *et al*, 1980; Sokoloff *et al*, 1989). If the plasma glucose level is unstable, it is required that a modified operational equation is used (Appendix 2.) (Savaki *et al*, 1980).

Chapter 3. Material and Methods:

Animals

Male Long-Evans hooded rats (Brock University, St. Catharines, Ontario) weighing 270-320 gm were used. Before surgery, the animals were housed in group cages and maintained on a 12 hr light:dark cycle (8 am:8 pm) under controlled humidity and temperature conditions. Food (Standard Lab Chow) and water were available *ad libitum.*

Experimental design:

The research paradigm consisted of a 3 X 2 factorial design with three levels of the dose condition and two levels of restraint, totaling 6 experimental groups (Table 9).

Table 9. Research paradigm

Group	unrestrained	restrained
Vehicle	8	8
BN 0.1µg	8	8
BN 0.5µg	8	8

Vehicle saline 3 µl.

By collapsing the two BN-treated groups and comparing them with controls, we tested for the existence of a BN treatment effect. By comparing low and medium dose conditions with control, we tested for a BN dose effect.

The restraint condition was designed to distinguish the effects of BN treatment on metabolic activities from those induced by motor stereotypy. If LCGU is affected in the unrestrained groups but not in the restrained groups, it would suggest that the alteration is a result of the execution of motor stereotypy. On the other hand, if the restrained and

unrestrained groups exhibit similar metabolic responses to BN, it would suggest that the alteration is not a result of the execution of motor stereotypy. Rather, the alterations is likely caused by BN treatment.

We tested for effects of restraint or BN upon LCGU by looking for an interaction between BN treatment and restraint. An interaction shows that the effects of BN upon cerebral metabolism differ over restraint conditions, and would prevent us from attributing alterations in functional activity to direct effects of BN.

Animals were randomly assigned to one of the following experimental groups (Table 9): (1) free-moving, 0.9% saline 3μl; (2) free-moving, BN 0.1μg/3μl; (3) free-moving, BN 0.5μg/3μl; (4) restrained, 0.9% saline 3μl; (5) restrained, BN 0.1μg/3μl; and (6) restrained, BN 0.5μg/3μl. The dose conditions were chosen according to published studies (for example, Merali and Piggins, 1990). At the 0.5 μg level, BN can elicit intensive grooming behaviors. Higher levels do not further enhance the behavioral effects (Rasler 1984).

Preparation of animals

Rats were weighed and rectal temperature was recorded prior to surgery. Inhalational general anesthesia was induced with 4% halothane carried in 66% nitrous oxide and 33% oxygen, and maintained with 1.5% halothane in 66% nitrous oxide and 33% oxygen.

Femoral vessel catheterization. A small incision was made in the skin of the right femoral triangle, at the level of the inferior edge of the epigastric muscle. A blunt dissection separated subcutaneous connective tissue, and a 1.5 cm length of the femoral vessels, just distal to the epigastric muscle, was isolated with 2 pairs of 7.5 cm curved, blunt

forceps. The same forceps were used to separate the femoral nerve from the artery. Three lengths of thread (3/0 silk, Ethicon, Somerville, NJ) were placed, one at a time, under each vessel. At the distal end of the dissection the vessel was tied off and the thread firmly taped to the surgery table. At the medial end of the dissection, the weight of a hemostat resting on the surgery table applied slight tension to another thread, temporarily occluding the flow of blood. The third thread remained unused at this time.

Microdisection scissors (7.5 cm) were used to cut a small triangular opening, extending about one-third the diameter of the vessel, in each vessel. A catheter of polyethylene tubing (PE 50, Intramedic Tubing, Clay-Adams, Parippany, NJ) filled with 0.9% saline solution (to which sodium heparin, 100 units/ml, was added to prevent blood coagulation within the tubing) and connected to a 3 ml plastic syringe, also filled with 0.9% saline solution, was then inserted into each vessel. After being advanced 3 to 4 cm, the catheter was secured in each vessel with the three threads.

After securing the intravascular catheters, a 0.5 cm incision was made in the skin at the nape of the neck between the scapulae, and a hypodermic needle was inserted into the femoral incision and advanced subcutaneously to the nape incision around the hind limb. The catheters were passed through the hypodermic needle and drawn through the nape incision. The catheters were flushed with 0.9% saline, containing sodium heparin (100 units ml^{-1}) to prevent blood coagulation within the tubing, and plugged with copper wire. The femoral and cervical incision were closed, and the catheters anchored to the skin at the neck with silk suture. A small piece of masking tape held the exposed portion of the catheters (about 4 cm) in a coil at the neck.

Third ventricle cannula implantation. Rats were mounted in a Kopf stereotaxic instrument with the incisor bar at 3.3 mm below interaural zero to achieve a flat skull. General anesthesia was maintained using the same combination of anesthetics through a modified plastic mask fitted onto the incisor bar. Coordinates for the third ventricle were 4.3mm from bregma, 0.0mm from the midline, 4.3mm ventral from the straight line passing through bregma and lambda (Paxinos and Watson, 1982; Merali and Piggins, 1990).

The point of cannula implantation was opened with a round head dental drill. A guide cannula (24 gauge, Plastic Products, Roanoke, VA) was implanted in the third ventricle. The guide cannula was then cemented to the skull with dental acrylic anchored to 4 jewelers screws. A stainless-steel obturator, extending 0.5 mm beyond the tip of the guide cannula, was kept in the guide cannula at all times except during injection.

The animals were allowed to recover in a wooden chamber maintained at 36°C until emergence. The animals were then returned to the animal room and housed individually in metal cages for further recovery. During the 48 hr recovery time the animals were maintained on the standard light/dark cycle with food and water available *ad libitum* until the experiment.

Peptide

BN (Pennisula Laboratories, Inc., CA) was obtained from Dr. Z Merali (School of Psychology, University of Ottawa). Each vial contained 50 µg BN, and was stored at -20°C with silica gel until use. For use, BN was dissolved with 0.9% saline (vehicle) at the following concentration: (1)

50μg in 1500μl 0.9% saline (0.1μg/3μl); and (2) 50μg in 300μl 0.9% saline (0.5μg/3 μl).

Experimental procedures

The animals were weighed and rectal temperature recorded. Those with post-surgical body weight loss over 25% and/or body temperature higher then 38.5°C were excluded from the experiment. The artery and vein catheters were extended with PE 50 tubing to 60 cm and secured to the cage. This allowed injection of isotope and blood sampling, while the animals moved freely. The animals were familiarized with the clear plastic test chamber (60 x 30 x 30 cm) padded with fine wood chips for at least 5 minutes, during which time blood samples were taken for baseline plasma glucose and hematocrit. Those with hematocrit lower then 50% were eliminated from the study. Lower hematocrit is caused by loss of large amount of blood during third ventricle cannulation.

The obturator was removed from the ventricular cannula immediately before injection, and the injector was inserted into the guide cannula. The injection tip extended 0.5 mm beyond the tip of guide cannula within the third ventricle. Injection was carried out with a hand held 25 μl microsyringe (Hamilton Co., Reno, NV) at a steady rate for about 1.5 to 2 minutes. The obturator was replaced in the guide cannula after the injection. Behavior was then observed for 5 minutes. Of the animals receiving BN, only those which exhibited stereotypic grooming behaviors within 5 minutes after injection proceeded further in the experiment.

For the unrestrained animals, a computer data logging program (DataLog, courtesy of Dr. Z Merali) was used to score animal behavior throughout the one hour experimental period, *i.e.* 15 minutes after ICV

injection, and 45 minutes 2DG incubation period. Animal behaviors were scored in 10 second epochs, according to the definition given in Table 10.

Table 10. Behaviors assessed and their operational definitions

Behavior	Operational definition
Head washing	The animal licks it's forepaws and wipe them over the face and crown regions
Head scratching	The side of the face or nape region is contacted by hind limbs
Body washing	The animal licks the ventral and/or lateral surfaces of the thorax, abdomen or tail
Body scratching	The flanks of the body are contacted by hindlimbs
Anal-genital	The animal licks the genital and/or anal regions
Locomotor behavior	The animal moves about the cage or actively orients to some aspects of the cage
Rest	The animal remains stationary or sleeps

(Modified from Merali and Piggins, 1990)

In order to distinguish BN LCGU effects on neural metabolism from those induced by bodily movement of behavioral stereotypy, a complete immobilization is required. To establish the restraint condition, immobilization was performed with a specially designed restraining jacket (Figure 3), and animals were further wrapped in a hand towel.

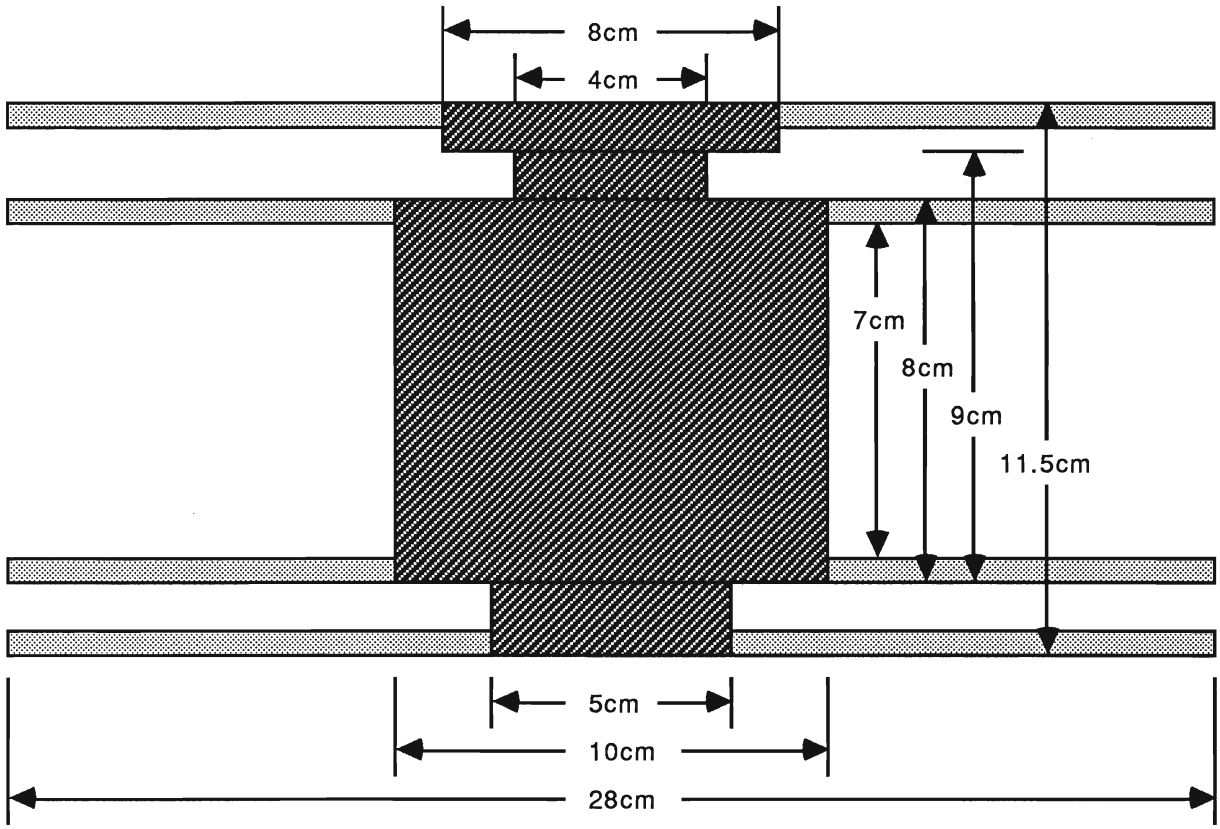

Figure 3. Restraining jacket with measurements (Shown smaller than actual size).

Measures were taken to ensure unrestricted breathing. A certain degree of head and forelimb movement was possible but any locomotion and grooming behavior was completely prohibited. Restrained animals were immobilized for 15 minutes before initiation of the 2DG experiment to match the time course used in freely moving animals.

2DG (100 μCi kg^{-1} in heparinized 0.9% saline) was injected through the venous catheter. Blood samples were taken in 50 μl capillaries (Fisher Scientific, Pittsburgh; PA) at 0, 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 10, 15, 20, 30, and 45, minutes and spun in a centrifuge (Brinkmann Inc.) for 3 minutes, after which hematocrit was measured (packed red blood cell volume/total blood volume). Plasma (20 μl aliquots) was taken from the centrifuged samples, mixed with 7 ml of scintillation fluid (Scintiverse, E. Fisher) and counted in a Beckman model 1800 liquid scintillation

counter (Beckman Instrument Inc., Fullerton, CA). Counting efficiency was determined and quench correlation was performed.

Additional blood samples (30 - 40 μl) were collected in Eppendorf microvials at 0, 1, 2, 3, 4, 5, 10, 15, 20, 30, and 45 minutes, and spun in a centrifuge (Brinkmann Inc.). In these samples, plasma glucose was enzymatically assayed using a Beckman Glucose Analyzer-2 (Beckman Instrument Inc., Fullerton, CA).

After the 45 minute 2[^{14}C]-DG incubation period, the animals were killed with a lethal dose of sodium pentobarbital delivered via the arterial catheter. The animals were decapitated with a small animal decapitator, and the brain was extracted from the skull within 5 minutes and frozen in 2-methylbutane chilled to -45 to -60°C on dry ice. The time interval from the 45 minute sample to complete freezing of the brain was within 8 minutes. The extracted brains were wrapped in aluminum foil, placed in a small glass jar filled with dry ice, and stored at -80°C until sectioning.

For sectioning, the brains were mounted on an object holder with M-1 embedding matrix (Lipshow, Mich.) and cut at 20 microns on a cryostat (Riechert-Jung, Model 3200A, Germany) within 36 hours after the run. The chamber temperature of the cryostat was maintained at -20 to -22°C; the temperature of the object holder was -14 to -16°C. Sections were picked up on hot (60°C) micro slides. The slides were placed on a hot plate (60°C) under a hair dryer. They were left under these conditions of moderate heat and warm air for at least 30 seconds. This procedure was taken to reduce isotope diffusion during the drying process. All the slides remained on the hot plate for at least 6 minutes.

Image analysis

The sections were then placed in contact with Kodak SB-5 blue X-ray film (SB-5, Kodak, Rochester; CN) for 20-24 days with [^{14}C]methylmethacrylate standards (Amersham). Following development of the film, autoradiographs were analyzed for LCGU using a computer image analyzer (MCID, M1; Imaging Research, Inc., St. Catharines, ON). The rate and lump constants were those described for the conscious rat (Sokoloff *et al*, 1989). Blood integrals were calculated with the modified 2DG method (Savaki *et al*, 1980).

Statistical analyses

The datalogging data were normalized using the total animal activity score (grooming/total, locomotion/total, rest/total), and expressed as a percentage of total activity for further processing.

The normalized behavioral data during the 1 hr experiment period, blood glucose data before and after [^{14}C]2DG injection, and LCGU data were analyzed using analysis of variance (ANOVA) on SPSS-PC+, version 2.0.

Two-way ANOVA was employed to test for overall main effects (BN treatment, restraint) and interaction. A BN treatment effect indicates that a difference in LCGU exists among BN treatment conditions. A restraint effect signifies that a difference in LCGU exists among the restraint conditions. An interaction effect indicates that the BN treatment effect is dependent upon the restraint conditions, or *vice versa*.

Multiple comparisons for one-factor (Group 1-6; one-way ANOVA) were used to compare individual groups. The Tukey's honestly significant difference (HSD) was used. In this analysis, the difference of treatment is

the only variable. The results of this analysis indicate the existence of a difference between individual groups.

One-way ANOVA for repetitive measurements was used to compare blood glucose changes before and after BN administration.

Chapter 4. Results

Animal physical conditions

We did not observe significant differences between any BN treatment and restraint conditions in pre-surgery body weight, pre-surgery body temperature, surgery time, recovery time allowed, pre-experiment body weight, and pre-experiment body temperature ($p>0.05$, two-way ANOVA) (Table 11). There was no interaction effect between any of the experimental conditions either.

Table 11. Physical conditions of the experimental animals (Mean±S.E.; n=8 unless missing value is indicated.)

Physical Conditions		Vehicle Unrestr.	BN 0.1μg Unrestr.	BN 0.5μg Unrestr.	Vehicle Restr.	BN 0.1μg Restr.	BN 0.5μg Restr.
Pre-surgery Wt.	gm	301.00 ±3.57	297.50 ±4.67	297.13 ±5.88	299.88 ±4.33	294.00 ±4.26	299.00 ±6.48
Pre-Surgery Temp.	℃	37.03 ±0.17	36.96 ±0.17	37.23 ±0.15	37.21 ±0.13	37.24 ±0.29	37.31 ±0.15
Surgery Time	min	103.75 ±07.95	133.13 ±12.10	105.00 ±06.34	108.75 ±08.39	105.63 ±04.57	124.38 ±09.13
Recovery Duration	hr	63.92 ±8.65	47.49 ±3.92	47.75 ±2.97	49.85 ±4.37	48.14 ±2.57	48.58 ±4.12
Pre-experiment Wt.	gm	277.50 ±5.55	265.13 ±6.33	269.38 ±7.54	268.88 ±5.04	259.38 ±4.64	268.63 ±5.05
Pre-experiment Temp.	℃	37.46 ±0.09	37.46 ±0.22	37.64 ±0.16	[m]37.7 ±0.15	37.79 ±0.16	37.49 ±0.16

[m] One missing value.

Behavioral observation

The distribution of behaviors the unrestrained vehicle group is shown in Table 10. Locomotor behaviors were coordinated without agitation. Occasional grooming activities were well coordinated. In most cases grooming included head washing and body licking, and head and body scratching. Exploring and grooming were separated by intervals where animals showed no obvious physical movement (resting). The inactive period may be followed by sleeping (typical sleeping position, eyes closed).

In the BN (0.5μg/3μl) unrestrained group, animals were in a constantly active state, except for brief rest periods. Locomotion was agitated (Table 12). Not all of the exploring activities appeared coordinated; a good part of the locomotor activities occurred in intervals between bursts of grooming behaviors. Locomotor behaviors also included chewing behavior and "wet dog shakes". Grooming appeared in abrupt bursts, which were frequently interrupted by brief locomotion or rest episodes. The rest period also seemed to be divided into two episodes: rest followed by overt grooming, and locomotor behaviors; and short pauses during vigorous grooming. In the BN 0.1μg group (Table 12) treated unrestrained group, behavior was similar to the BN 0.5μg group, while grooming and locomotor behaviors were manifested at a lower level (Table 12). Sleep was absent in both of the BN treated groups.

BN treatment increased total grooming time and decreased time spent resting during the 1 hr observation period ($p<0.01$, ANOVA) (Table 12). Using multiple comparison techniques, difference between individual groups was identified. They are shown in Table 12.

Most animals in the three restrained groups appeared quiet after BN injection. Some of the saline treated animals even had observable sleep periods (complete quiet, eyes closed). In all of the BN treated animals, there were occasional attempts to move the neck and the forepaws. Occasional episodes of struggling could be seen in all animals. No grooming behaviors were observed.

Table 12. Effects of BN on behavior (Mean±S.E.; n=8.)

Behavior	Groups		
	Vehicle	BN 0.1μg	BN 0.5μg
Grooming	04.74±0.90	25.05±3.36**	46.09±3.88**,††
Locomotor behaviors	47.71±4.61	32.22±4.60*	30.65±2.27**
Rest	47.56±4.84	42.77±2.17	22.81±2.63**,††

* p<0.05, ** p<0.01 comparing with vehicle;
† p<0.05, †† p<0.01 comparing with BN 0.1 μg.

Blood Glucose Measurement

Blood glucose data before and after ICV injection are shown in Table 13, and Figure 4. There was no significant BN treatment effect nor restraint effect ($p>0.05$; two-way ANOVA) in the zero time sample before BN ICV injection (base line) among all of the experimental conditions (Table 13).

In the two vehicle groups, unrestrained animals showed a significant decrease in blood glucose after ICV injection ($p<0.01$; one-way ANOVA) (Table 13; Figure 4) due to food deprivation during experiment. In the two BN 0.1μg groups, BN injections caused significant increase in blood glucose in both restraint conditions ($p<0.01$; one-way ANOVA) (Table 13; Figure 4). The two BN 0.5μg groups showed similar changes as in BN 0.1 groups (Table 13; Figure 4).

Table 13. Effect of BN on blood glucose level (mg/dl)
(Mean±S.E.; n=8 unless missing value is indicated.)

Time	Vehicle Unrestr.	BN 0.1 μg Unrestr.	BN 0.5 μg Unrestr.	Vehicle Restr.	BN 0.1μg Restr.	BN 0.5 μg Restr.
Before ICV injection	150.29[m] ±4.22	148.88 ±4.38	145.71[m] ±3.19	154.57[m] ±2.97	155.00 ±5.42	153.25 ±3.72
After ICV injection	135.24§ ±0.90	187.32** ±2.49	202.25** ±2.70	154.69 ±2.15	187.72** ±4.29	205.12** ±2.83

[m] One missing value.
§ $p<0.01$ comparing with the blood glucose levels before intracerebroventricular injections (lower);
** $p<0.01$ comparing with the blood glucose levels before intracerebroventricular injections (higher).

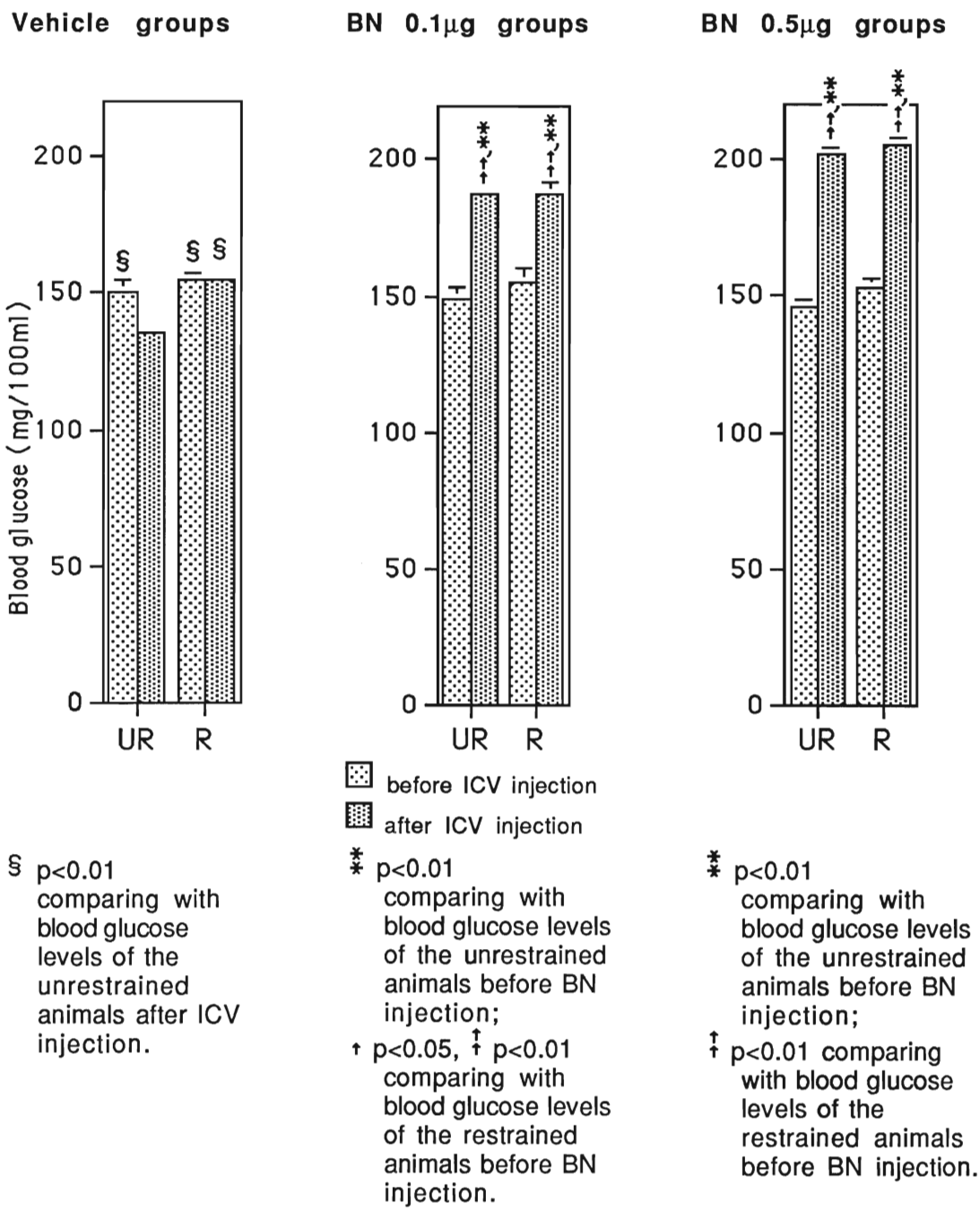

Figure 4. Comparison of blood glucose before and after ICV injection.

Abbreviations: UR: unrestrained; R: restrained.

Whole brain glucose utilization

We did not observe any effect of BN treatment or restraint on overall cerebral metabolism rate (WB CGU) ($p>0.05$; two-way ANOVA) (Table 14).

Table 14. BN effect on whole brain glucose utilization (WB CGU, μmol 100 g^{-1} min^{-1}) (Mean±S.E.; n=8.)

Structures	Vehicle Unrestr.	BN 0.1μg Unrestr.	BN 0.5μg Unrestr.	Vehicle Restr.	BN 0.1μg Restr.	BN 0.5μg Restr.
WB	42.04 ±1.88	41.12 ±2.09	37.30 ±1.13	38.45 ±2.05	39.14 ±4.23	39.19 ±2.11

Local cerebral glucose utilization Measurement

Across the three categories, there was no interaction between the effects of BN dose and the effects of restraint on local cerebral glucose utilization (LCGU) ($p>0.05$, two-way ANOVA). Data from the same BN treatment conditions (unrestrained and restrained) were therefore collapsed into three BN treatment groups, *i.e.* vehicle, BN 0.1μg, BN 0.5μg to test for BN treatment effects. Similarly, data from all the three BN treatment conditions were collapsed to test for restraint effects.

Primary Regions. Collapsed data show that, among the four anterior thalamic subdivisions (our primary category), the anteroventral thalamic nucleus (AV) ($p=0.029$, two-way ANOVA), and the anteroventral thalamic nucleus, ventral lateral portion (AVVL) ($p<0.0005$, two-way ANOVA) exhibited significant response to BN treatment (Table 15; Figures 5, 6). However, no main effect for restraint or interaction effects between BN treatment and restraint was observed in these two regions ($p>0.05$, two-way ANOVA). Diagrams of the rat brain coronal section show our sampling locations (Figure 7).

Table 15. BN treatment effect on LCGU: Primary structures

Structures	BN treatment effect*	Restraint effect†
AD	-	-
AM	-	-
AV	+	-
AVVL	+ + +	-

+ $p<0.05$, ++ $p<0.01$, +++ $p<0.001$.
Abbreviations:
AD: anterodorsal thalamic nucleus;
AM: anteromedial thalamic nucleus;
AV: anteroventral thalamic nucleus;
AVVL: anteroventral thalamic nucleus, ventral lateral portion.
* Data were collapsed across the two restraint conditions and the two dose conditions (0.1 μg and 0.5 μg) to show BN treatment effects (two-way ANOVA);
† Data were collapsed across the three dose conditions (vehicle, BN 0.1 μg and 0.5 μg) to show restraint effects (two-way ANOVA).

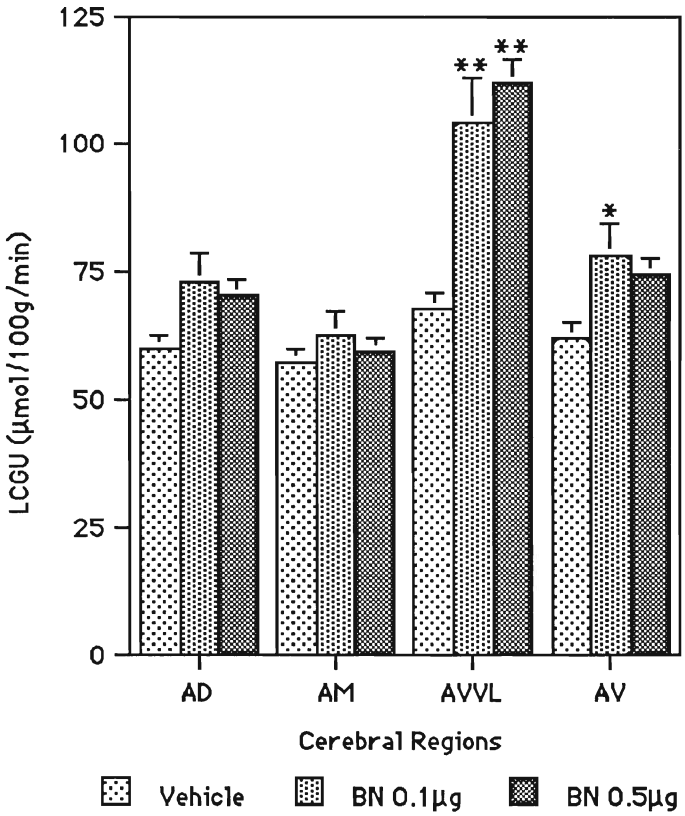

Figure 5. Primary regions: AD, AM, AVVL, AV. LCGU for vehicle, BN 0.1 µg, BN 0.5 µg groups. Data are collapsed across two restraint conditions into three dose conditions to compare BN treatment effects.

* $p<0.05$, ** $p<0.01$ comparing with vehicle groups.

Abbreviations:

AD: anterodorsal thalamic nucleus;
AM: anteromedial thalamic nucleus;
AV: anteroventral thalamic nucleus;
AVVL: anteroventral thalamic nucleus, ventral lateral portion.

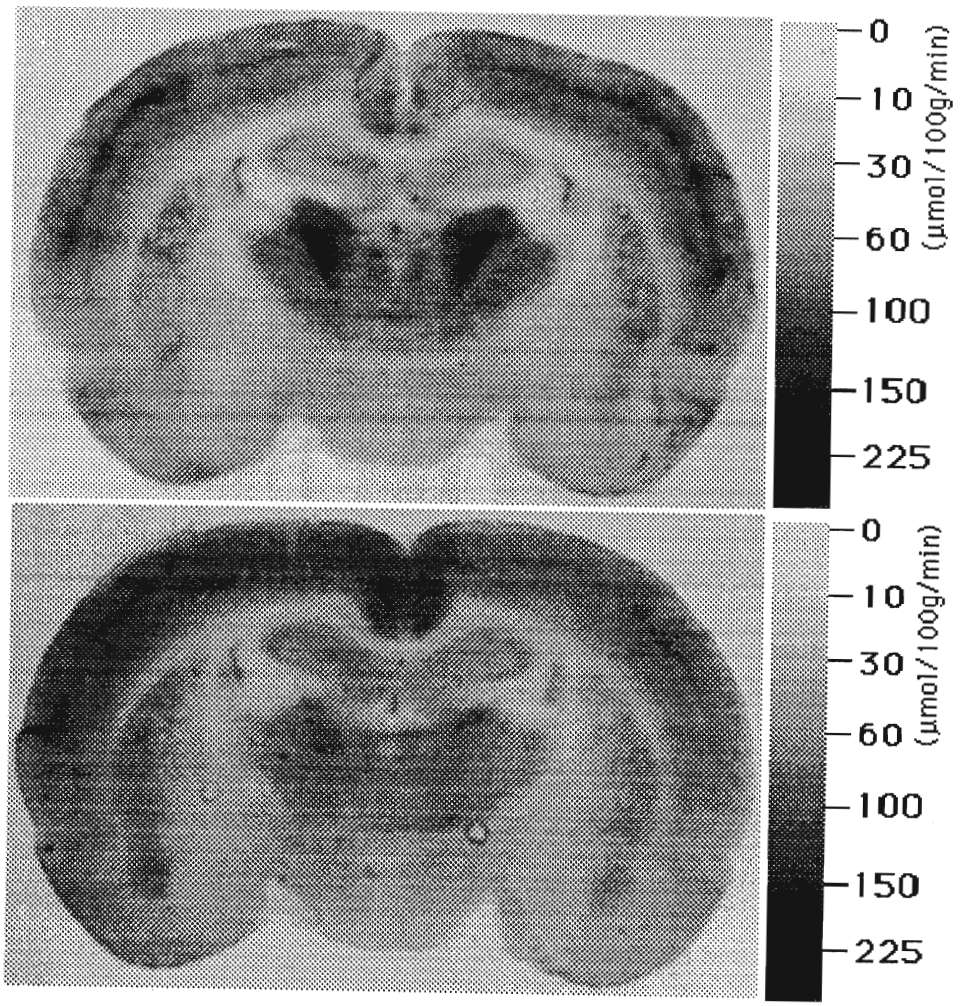

Figure 6. Gray scale [^{14}C]2DG image illustrating increased LCGU in anteroventral thalamic nucleus and its ventral lateral portion after BN administration. The top image represents BN 0.5 µg, unrestrained animals; the bottom image represents vehicle, unrestrained animals.

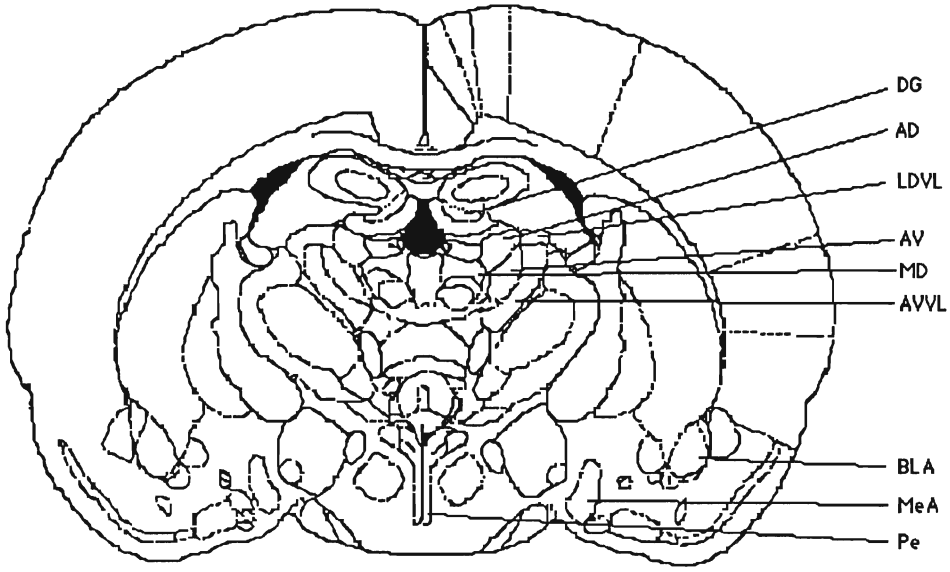

Figure 7. Coronal section of the rat brain at the level of the anterior thalamus, showing the sampling locations for AV, AD, AVVL, LDVL (Interaural 7.20 mm, Bregma 1.80 mm) (Modified from Paxinos and Watson, 1989).

Abbreviations:

AD: anterodorsal thalamic nucleus;
AM: anteromedial thalamic nucleus;
AV: anteroventral thalamic nucleus;
AVVL: anteroventral thalamic nucleus, ventral lateral portion;
BLA: basolateral amygdaloid nucleus, anterior part;
DG: dentate gyrus;
LDVL: lateral dorsal thalamic nucleus, dorsal medial;
MD: mediodorsal thalamic nucleus;
MeA: medial amygdaloid nucleus;
Pe: periventricular hypothalamic nucleus;
SCh: suprachiasmatic nucleus.

Multiple comparison techniques (Tukey HSD) revealed alterations in LCGU caused by differences in individual experimental conditions in AV and AVVL (Figures 8, 9).

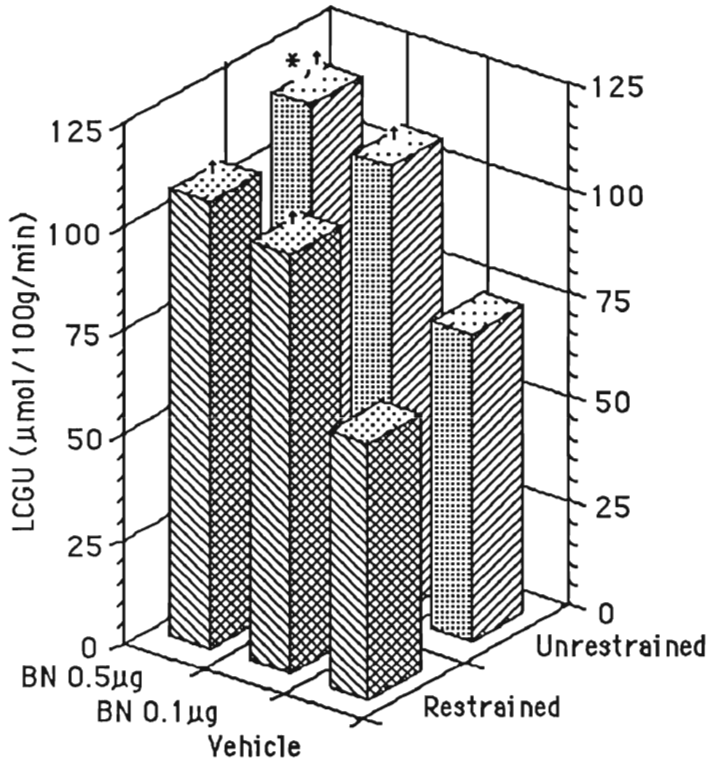

Figure 8. Anteroventral thalamic nucleus, ventral lateral portion: LCGU for unrestrained and restrained animals treated with BN 0.5 µg, BN 0.1 µg, and vehicle.
* $p<0.05$ comparing with unrestrained vehicle group;
† $p<0.05$ comparing with restrained vehicle group.

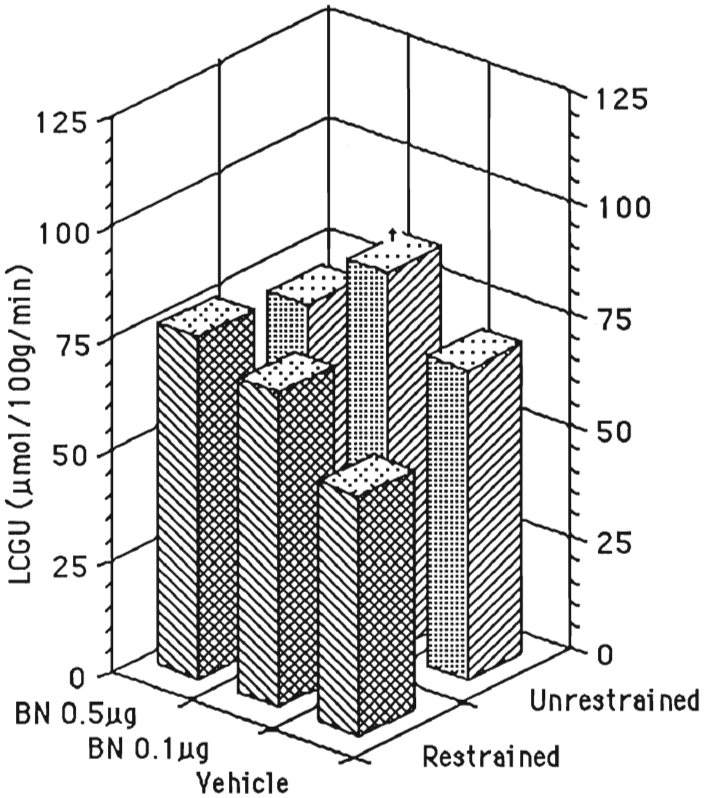

Figure 9. Anteroventral thalamic nucleus: LCGU for unrestrained and restrained animals treated with BN 0.5 µg, BN 0.1 µg, and vehicle.
† $p<0.05$ comparing with restrained vehicle group.

Secondary Regions. Data collapsed across the restraint conditions show a BN treatment effect in the internal granular layer of the olfactory bulb (IGr) and the suprachiasmatic nucleus (SCh) ($p=0.028$, $p=0.003$, respectively, two-way ANOVA) (Table 16; Figures 10, 11). No restraint effect or interaction effect was found in these regions. Diagrams of the rat brain coronal section show our sampling locations (Figures 12, 13).

Table 16. BN treatment effect on LCGU: Secondary structures

Structures	BN treatment effect*	Restraint effect†
IGr	+	-
Mi	-	-
Tu	-	-
NcDL	-	-
Acb	-	-
DG	-	-
S	-	-
ST	-	-
BLA	-	-
La	-	-
MeA	-	-
PMCo	-	-
MD	-	-
Pe	-	-
SCh	+ +	-
LC	-	-
Sol	-	-
SubGel	-	-

+ $p<0.05$, ++ $p<0.01$, +++ $p<0.001$.
Abbreviations: (See page 6; Appendix I).
* Data were collapsed across the two restraint conditions and the two dose conditions (0.1 µg and 0.5 µg) to show BN treatment effects (two-way ANOVA);
† Data were collapsed across the three dose conditions (vehicle, BN 0.1 µg and 0.5 µg) to show restraint effects (two-way ANOVA).

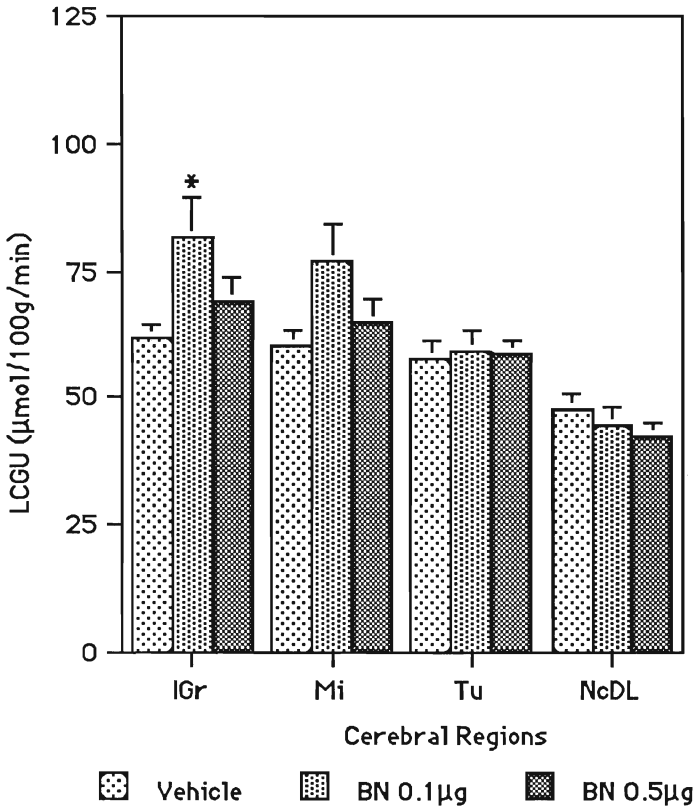

Figure 10. Secondary regions: IGr, Mi, Tu, NcDL. LCGU for vehicle, BN 0.1 µg, BN 0.5 µg groups. Data are collapsed across two restraint conditions into three dose conditions to compare BN treatment effects.

* $p<0.05$, ** $p<0.01$ comparing with vehicle groups.

Abbreviations:

IGr: internal granular layer of the olfactory bulb;
Mi: mitral cell layer of the olfactory bulb;
Tu: olfactory tubercle;
NcDL: deep layer of neocortex.

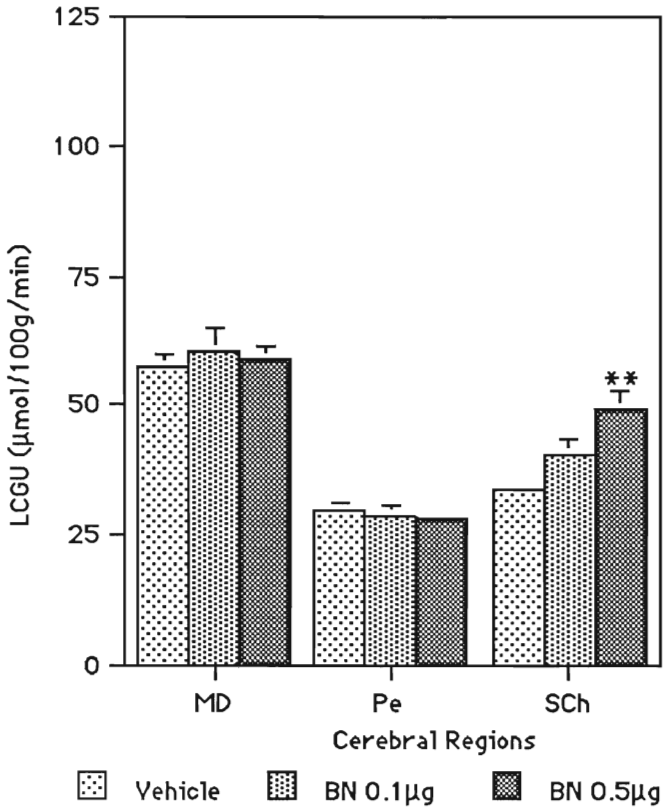

Figure 11. Secondary regions: MD, Pe, SCh. LCGU for vehicle, BN 0.1 µg, BN 0.5 µg groups. Data are collapsed across two restraint conditions into three dose conditions to compare BN treatment effects.

* $p<0.05$, ** $p<0.01$ comparing with vehicle groups.

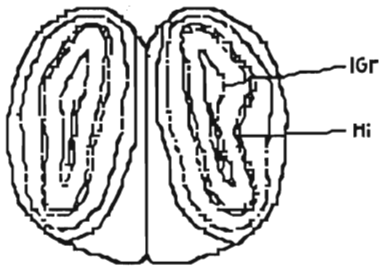

Figure 12. Coronal section of the rat brain at the level of the olfactory bulb, showing the sampling locations for IGr, and Mi (Interaural 15.70 mm, Bregma 6.70 mm) (Modified from Paxinos and Watson, 1989).
Abbreviations:
IGr: internal granular layer of the olfactory bulb;
Mi: mitral cell layer of the olfactory bulb.

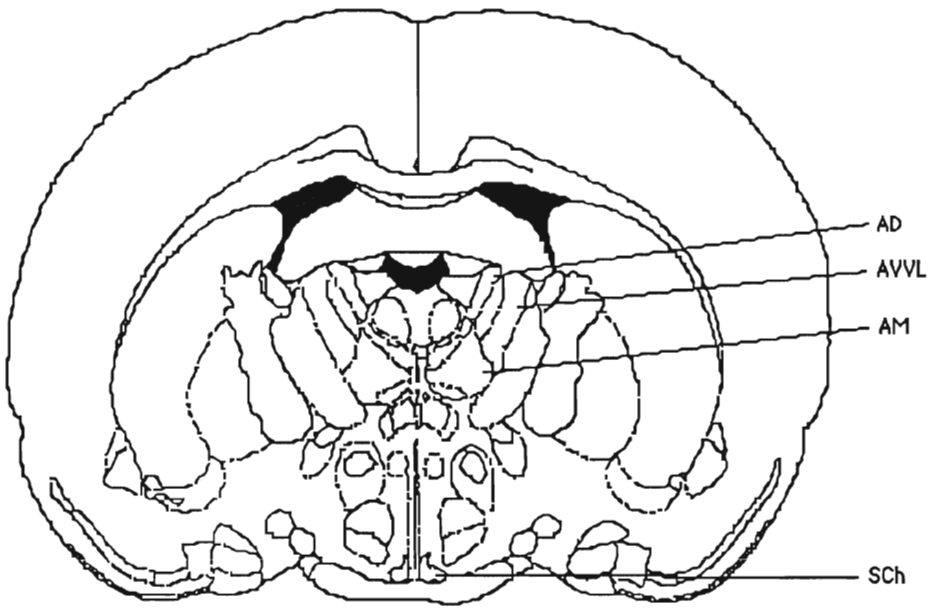

Figure 13. Coronal section of the rat brain at the level of the anterior thalamus, showing the sampling location for SCh (Interaural 7.60 mm, Bregma 1.40 mm) (Modified from Paxinos and Watson, 1989).
Abbreviations:
AD: anterodorsal thalamic nucleus;
AM: anteromedial thalamic nucleus;
AVVL: anteroventral thalamic nucleus, ventral lateral portion;
SCh: suprachiasmatic nucleus.

Tertiary Regions. Data collapsed across the restraint conditions show a significant treatment effect in the median eminence (ME) ($p=0.011$; two-way ANOVA) (Table 17, Figure 14). No main effects for restraint or interaction effect was found in this region ($p>0.05$, two-way ANOVA). Diagrams of the rat brain coronal section show our sampling locations (Figure 17).

Table 17. BN treatment effect on LCGU: Tertiary structures

Structures	BN treatment effect*	Restraint effect†
Cg1	-	-
Cg2	-	-
Cg3	-	-
Hi	-	-
Ent	-	-
LDDM	-	+
LDVL	-	+
VL	-	-
VM	-	-
VPL	-	-
VPM	-	-
LG	-	+
VMH	-	-
DM	-	-
LH	-	-
PH	-	-
Arc	-	-
InfS	-	-
ME	+	-
MM	-	-
LM	-	-
3	-	-
SC	-	-

+ $p<0.05$, ++ $p<0.01$, +++ $p<0.001$.
Abbreviations: (See Page 7; Appendix I).
* Data were collapsed across the two restraint conditions and the two dose conditions (0.1 µg and 0.5 µg) to show BN treatment effects (two-way ANOVA);
† Data were collapsed across the three dose conditions (vehicle, BN 0.1 µg and 0.5 µg) to show restraint effects (two-way ANOVA).

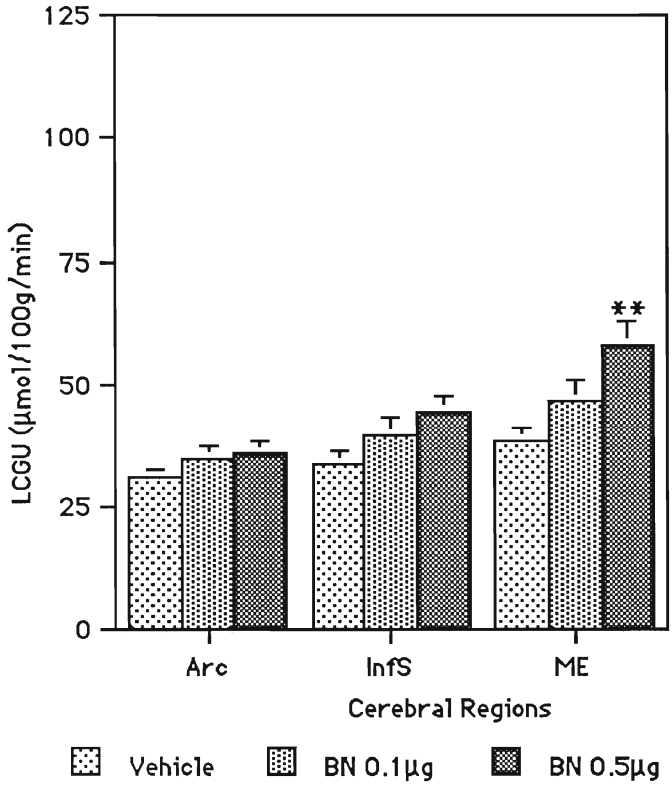

Figure 14. Tertiary regions: Arc, InfS, ME. LCGU for vehicle, BN 0.1 µg, BN 0.5 µg groups. Data are collapsed across two restraint conditions into three dose conditions to compare BN treatment effects.

* $p<0.05$, ** $p<0.01$ comparing with vehicle groups.

Abbreviations:
Arc: arcuate hypothalamic nucleus;
InfS: infundibular stem;
ME: median eminence.

Multiple comparison techniques (Tukey HSD) revealed alterations in LCGU caused by differences in individual experimental conditions in ME (Figure 15).

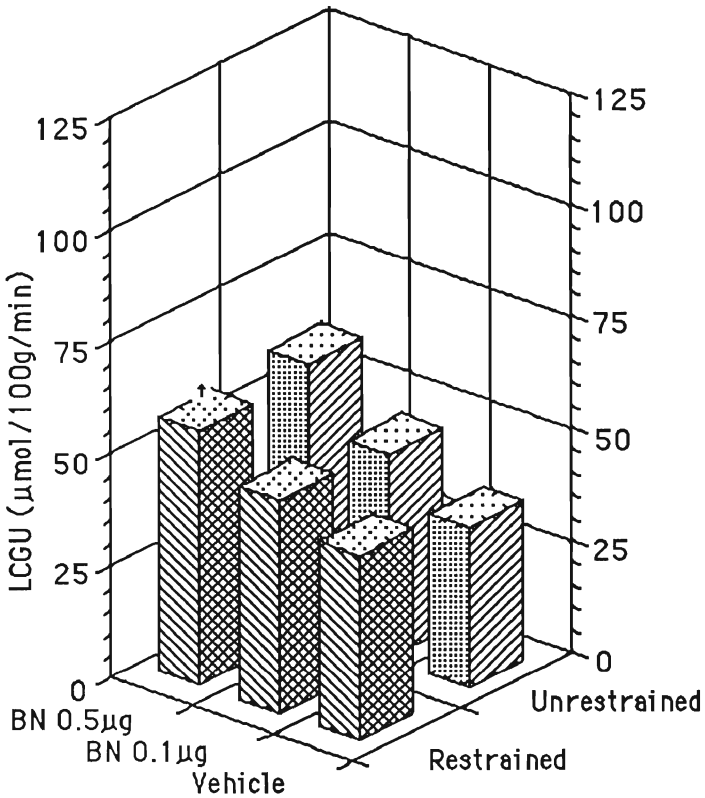

Figure 15. Median eminence (ME): LCGU for unrestrained and restrained animals treated with BN 0.5 µg, BN 0.1 µg, and vehicle.
† $p<0.05$ comparing with restrained vehicle group.

Based on data collapsed across the three dose conditions, a restraint effect was found in both of the laterodorsal thalamic nucleus (LD) subdivisions, including its dorsomedial portion (LDDM) ($p=0.044$) and ventrolateral portion (LDVL) ($p=0.009$). A restraint effect was also found in lateral geniculate (LG) ($p=0.027$) (two-way ANOVA) (Table 17, Figure 16). No main effect for BN treatment or interaction between BN treatment and restraint was found in these regions ($p>0.05$, two-way ANOVA). Diagrams of the rat brain coronal section show our sampling locations for

LDDM, and LDVL (Figure 17).

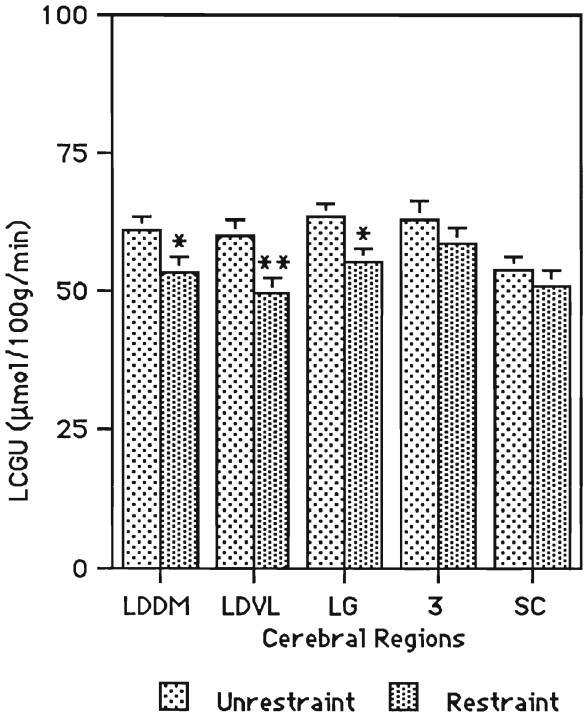

Figure 16. Tertiary regions: LDDM, LDVL, LG, *3*, SC. Data are collapsed into two restraint conditions to compare restraint effects.
* $p<0.05$, ** $p<0.01$ comparing with unrestrained groups.
Abbreviations:
LDDM: lateral dorsal thalamic nucleus, dorsal medial;
LDVL: lateral dorsal thalamic nucleus, ventral lateral;
LG: lateral geniculate;
3: oculomotor nucleus;
SC: superior colliculus.

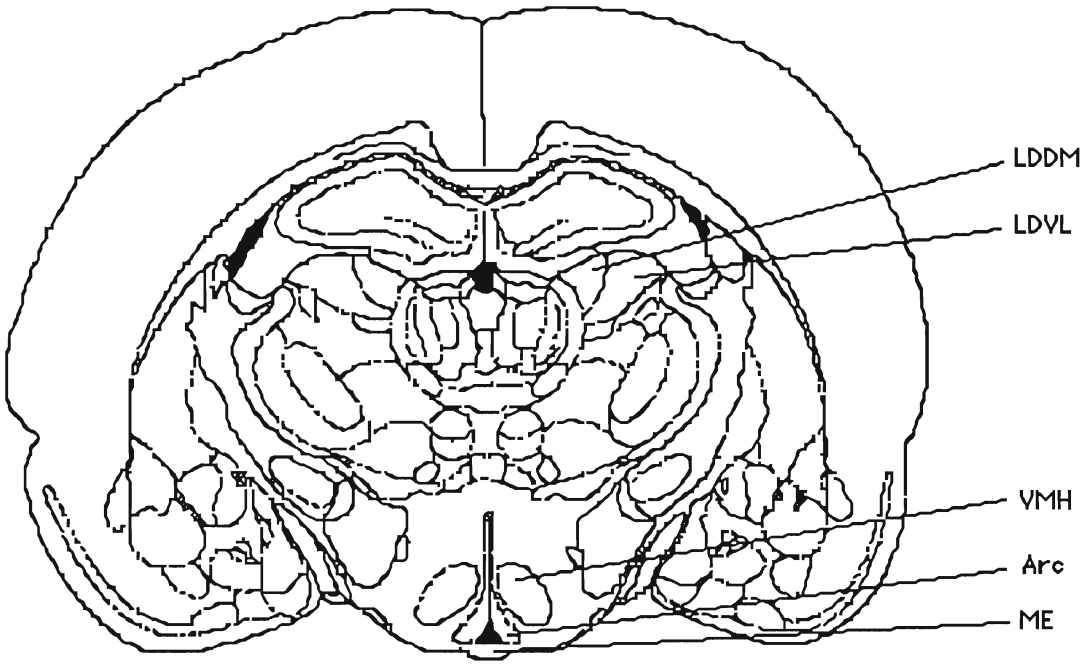

Figure 17. Coronal section of the rat brain at the level of the laterodorsal thalamus, showing the sampling locations for LDDM, LDVL, ME (Interaural 6.20 mm, Bregma 2.80 mm) (Modified from Paxinos and Watson, 1989).

Abbreviations:

Arc: arcuate hypothalamic nucleus;
LDDM: lateral dorsal thalamic nucleus, dorsal medial;
LDVL: lateral dorsal thalamic nucleus, ventral lateral;
ME: median eminence;
VMH: ventromedial hypothalamic nucleus.

Chapter 5. Discussion

The main objective of our study was to localize the functional effects of BN within the CNS. A large number of regions were mapped with the [^{14}C]2DG method, and we had to conduct statistical tests for each region. Essentially, each region was an independent experiment. Therefore, it was possible that we would come across some significant findings purely by chance. In order to minimize the chance of type I error in the regions we were most interested in, we organized cerebral regions into three categories, namely *the primary regions*, *the secondary regions*, and *the tertiary regions*. The primary regions were of most interest based on our previous findings of BN effects in the anterior thalamus. The secondary regions were of interest because they have been shown to have high concentrations of BN receptors. The tertiary regions were chosen based on the association of these regions with the limbic system, the thalamus, and the hypothalamus. Subcortical visual structures were also included due to their association with animal locomotion.

Using this categorization, we can decrease the number of regions tested within each category and, therefore decrease the chance of including a statistical artifact within the primary and secondary categories. Since the primary regions, in particular, only needed four tests, the *p* values can be examined with greater confidence that they are not a statistical artifact.

The anteroventral thalamic nucleus, especially its ventral lateral portion, shows increased metabolic activity following BN administration

We found strong BN treatment effects in AV, especially in AVVL. These effects were not influenced by behavioral restraint that completely prohibited the animals from exhibiting behavioral stereotypy. Therefore, in AV and AVVL, regional alterations in LCGU reflected BN influences upon cerebral function. They did not result from BN-induced behavioral stereotypy.

We have found no published data regarding BN receptor distribution in AVVL. Our observation of increased LCGU in AV, especially in AVVL, probably reflects BN-induced functional alterations arising elsewhere and projecting to the anterior thalamus. This suggestion of a remote origin for the observed effects should be examined in context of the neuroanatomy of the limbic circuitry and the hypothalamic regions, and the distribution of BN receptors.

One possible origin of projections to the anterior thalamus is the hypothalamus. As the principle limbic synaptic relay for information reaching the limbic cortex (Kelly and Dodd, 1991; Kupfermann, 1991a), the anterior thalamus receives projections from the mammillary bodies and hypothalamus via the mammillothalamic tract (tract of Vicq-d'Azyr) (MTT) (MacLean, 1986). It also receives neural projections directly from the hippocampus via the fornix and indirectly from the hippocampus via the fornix-mammilary complex pathway (Figure 18) (Faull and Mehler, 1985; Kupfermann, 1991a).

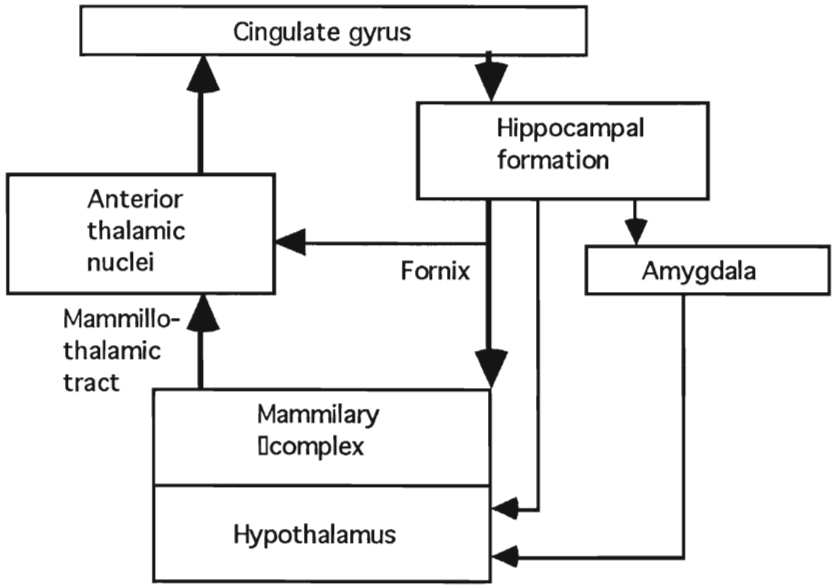

Figure 18. The limbic circuitry. The limbic circuit originally proposed by James Papez is indicated by thick lines; more recently described connections are shown by fine lines. (Modified from Kupfermann, 1991a).

Neural afferents from other limbic regions usually project to a specific subdivision of the anterior thalamus (Figure 19) (Isaacson, 1974). Hippocampal projections to the anterior thalamus pass via the fornix mainly to AV and AM. The mammillary complex projects via MTT to AV, AM and AD; in particular, the medial mammillary nucleus projects bilaterally to AD. Afferents from both the cingulate cortex and the retrosplenial granular cortex terminate in AV and AM (Faull and Mehler, 1985; Isaacson, 1974). AChE staining studies have shown that AVVL receives fiber projections from MTT, especially in the caudal ventromedial region where it is often dispersed by fiber fascicles from MTT (Figure 19) (Faull and Mehler, 1985). We have been unable to find data regarding synaptic projections to AVVL, and would suggest that further study of projections to this region might be useful in localizing BN's sites of action upon the CNS.

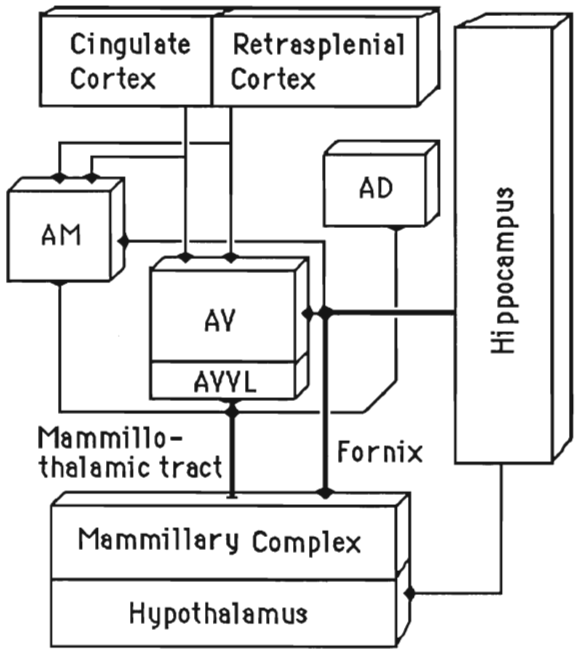

Figure 19. Neural projections to the anterior thalamus subdivisions. Thick lines indicate projections that may be involved in the transmission of BN-induced effects from the hippocampus, and hypothalamus; fine lines indicate projections to other subdivisions (From Faull and Mehler, 1985; Isaacson, 1974).
Abbreviation:
AV: anteroventral thalamic nucleus;
AM: anteromedial thalamic nucleus;
AD: anterodorsal thalamic nucleus;
AVVL: ventral lateral portion of AV.

It has been suggested that BN receptors, located in the hypothalamic regions, the hippocampus, and amygdala (Zarbin *et al*, 1985; Moody *et al*, 1988; Battey and Wada, 1991), mediate BN-induced behavioral stereotypy. Our data suggest that AV, especially AVVL should be added to the list structures involved in the mediation of behavioral stereotypy.

The strong influence of BN upon functional activity in the anterior thalamus may be linked to stereotypy. It has been demonstrated that animals with MTT lesions are slow or reluctant to begin new behavioral acts (Isaacson, 1974), indicating that the interruption of the neural pathway from the mammillary complex to the anterior thalamus

attenuates the initiation of novel behaviors. Since destruction of the MTT induces effects which are the opposite of BN-induced behavioral stereotypy, it is suggested that MTT may conduct BN-induced functional alterations from the hippocampus, amygdala, and hypothalamic regions to the anterior thalamus.

Increased metabolic activities in the ventral lateral portion of the anteroventral thalamic nucleus may reflect altered cholinergic activities in this region during alterations of limbic and hypothalamic functioning

AChE delineates fiber terminals that contain acetylcholine (Paxinos and Watson, 1989). AV displays a high level of AChE activity especially in its ventrolateral portion (AVVL), whereas AD and AM are characterized by, respectively, moderate and low levels of AChE activity (Faull and Mehler, 1985; Paxinos and Watson, 1989). AV is also characterized by its high cellular density; the cells are generally very closely packed, except in the caudal ventromedial region where they are often dispersed by fiber fascicles from MTT (Figure 19) (Faull and Mehler, 1985). High AChE activity and high density of fiber fascicles from MTT suggest that AVVL may contain a large cholinergic synaptic field, which receives neural terminals mainly from MTT.

Based on the histological composition of AV, especially its ventral lateral part, and our observations of BN-induced metabolic alterations in this region, it is suggested that metabolic alterations in AVVL reflect increased synaptic activity in this region. As maintenance of cellular homeostasis in association with EPSPs and/or IPSPs (the active pumps) are the major energy expenditure of the neurons, increased LCGU in AVVL reflects increased energy consumption caused by greater amount of EPSP and/or IPSP activities at the synapses within AVVL. Given the high

density of AChE activities in AVVL, it is further suggested that increased EPSP and/or IPSP in AVVL is mediated by cholinergic synapses.

Behavioral stereotypy induced by BN and other neural peptides may be mediated by the same neural circuitry

Other neuropeptides, *e.g.* ACTH, TRH, and SP, can also cause stereotypic grooming and locomotion when given centrally (Gmerek and Cowan, 1983; Cowan *et al*, 1985; Van Wimersama Greidanus *et al*, 1988; Meisenberg and Simmons, 1986). The fact that completely different peptides can cause a similar behavioral stereotypy that only differs in details suggests that various neural peptides induce behavioral stereotypy through a common neural circuitry.

Therefore, it would be of interest to study the central effects of ACTH, TRH, SP on cerebral metabolism in AV, and AVVL. If the central administration of these peptides can induce alterations of LCGU in AV, especially in AVVL, the suggestion that the behavioral stereotypy induced by these peptides is mediated by common neural circuitry would be supported.

Increased metabolic activities in the suprachiasmatic nucleus may reflect altered functional state of BN receptors in the nucleus and surrounding regions

We found that central BN administration caused increased metabolic activity in SCh. This effect did not interact with physical restraint. Therefore, the SCh findings represent direct BN effects, and not secondary manifestations of motor activity. It is likely that the effect results from BN interaction with the high MBN receptor density in SCh (Table 2).

As the primary biological clock in mammals, SCh functions as an internal circadian pacemaker (Kelly, 1991), and may be responsible for the maintenance of circadian rhythms of sleep, locomotion, feeding and drinking, and adrenal corticosterone secretion (Kelly, 1991; Bleier and Byne, 1985). All of these functions are affected by central BN administration.

Immunohistological studies have confirmed that SCh has mutual projections with surrounding hypothalamic regions, as well as some remote structures such as the thalamus and hippocampus. Some of these projections contain BN-like immunoreactivities (Watts and Swanson, 1987; Mikkelsen, *et al*, 1991). Because of the widespread presence of BN receptors in the hypothalamus and hippocampus (Battey and Wada, 1991), and because of reciprocal projections between SCh and these regions (Watts and Swanson, 1987), functional alterations in SCh may contribute to the disruption of circadian rhythms observed following BN administration.

Increased metabolic activities in the internal granular layer of the olfactory bulb may be caused by alterations of bombesin receptor function

IGr exhibited a BN-induced increase in LCGU. We failed to see a restraint effect or interaction between treatment and restraint. Therefore, the effects observed in IGr are caused by BN central administration and are a direct result of BN influence upon neural function.

Our findings of BN-induced metabolic alterations in IGr agree with *in vitro* [^{125}I-Tyr^{4}]BN autoradiographic binding studies showing high BN-receptor densities in the olfactory bulb (Table 2) (Zarbin *et al*, 1985; Moody *et al*, 1988). *In situ* hybridization studies performed with cDNA

specific probes demonstrated that NMB receptor mRNA and NMB mRNA expression was most prominent in the olfactory regions (Wada *et al*, 1991, Battey and Wada, 1991). Because of the high densities of NMB receptor distributions in the olfactory region, it is suggested that increased metabolism in IGr is the reflection of BN effects upon BN receptors in this region.

Increased metabolic activities in the median eminence may reflect bombesin effects on receptors localized on parvocellular neurons

ME exhibited a BN-induced increase in LCGU. We failed to see a restraint effect or interaction between treatment and restraint in ME. Therefore, the effects observed in ME are caused by BN central administration and are a direct result of BN influence upon cerebral function.

As a part of the pituitary portal system, ME receives projections from parvocellular neurons in the following hypothalamic regions: the paraventricular nuclei (or infundibular nucleus), periventricular region, medial basal region, arcuate and tuberal nuclei, and preoptic nuclei (Figure 20) (Bleier and Byne, 1985; Armstrong, 1985; Kupfermann, 1991a).

Among the hypothalamic regions that contain parvocellular neurons, the periventricular nucleus has been demonstrated to have high concentrations of ^{125}I-[Tyr^4]BN bindings (GRP and/or NMB receptors) (Moody *et al*, 1990; Zarbin *et al*, 1985). In addition, the paraventricular nucleus has been demonstrated to express GRP mRNA, and to express GRP receptor mRNA very strongly (Wada *et al*, 1990; Wada *et al*, 1991; Battey and Wada, 1991) (Figure 20). Projections of these regions to ME may underlie the effects we observed in this region.

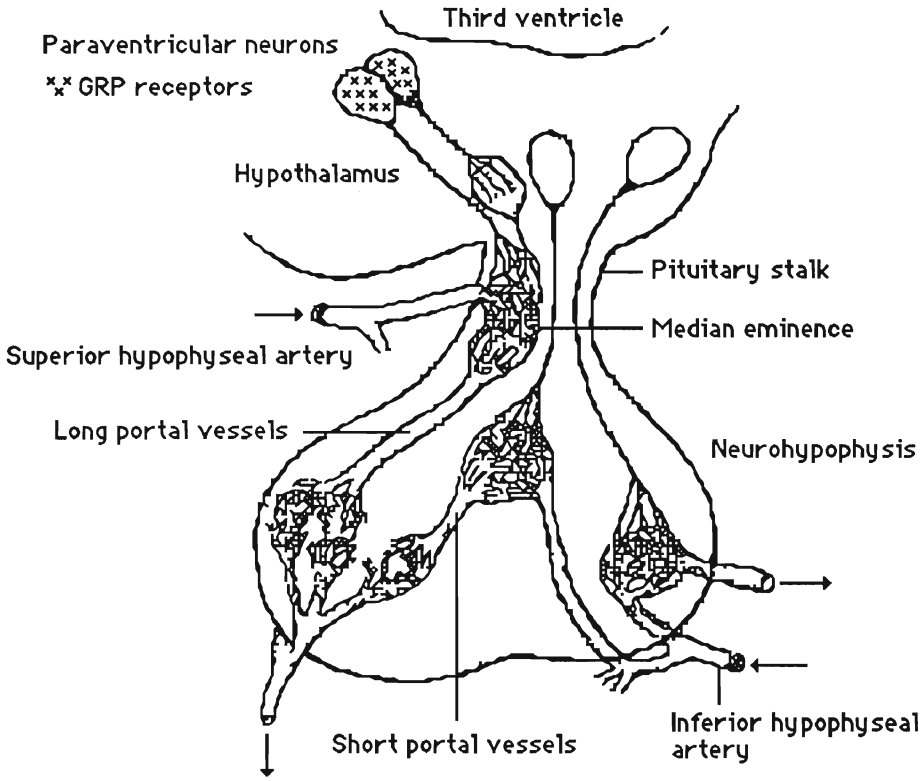

Figure 20. Role of GRP receptors in modulating parvocellular neuron releasing functions. ICV BN binds to GRP receptors on the parvocellular neuron surface. Stimulated neurons secrete releasing or inhibiting hormone in ME, or modulate secretion of another neuroendocrine neuron. (Modified from Kupfermann, 1991a).

Functional alterations of the hyppocampus after BN central administration could also have effects on endocrine functions, since autoradiography studies in the rat have provided evidence of fibers from the ventral subiculum reaching the "medial preoptic-anterior hypothalamus area" and terminating in the ventralmedial and arcuate nuclei (MacLean, 1985).

Central BN administration is associated with increased blood level of ACTH (Sander, 1991), GH (Bitar *et al*, 1991; Houben and Denef, 1990), and TSH (Malendowicz and Miskowiak, 1990). The releasing and inhibiting neurohormones or precursor peptides for these anterior pituitary hormones are synthesized in the parvocellular neurons and packaged in neurosecretory vesicles, and then transported down the axon to the

terminal, where they are stored and released by secretion to the ME portal vasculature (Figure 20) (Kupfermann, 1991a; Schwartz, 1991). Our findings that BN treatment increases LCGU in ME support the hypothesis that increased neural transportation and enhanced exocytosis in ME increases energy consumption in this region.

Dopamine is the only non-peptide inhibiting hormone released in ME. It has been shown that BN and GRP increase the concentration of dopamine metabolites in ME and in the intermediate lobe of the pituitary (Manzanares *et al*, 1991). Following BN administration, increased dopaminergic IPSPs in the tuberoinfundibular neurons may be a part of the increased metabolic rate in ME.

Physical restraint may inhibit emotional expression in the animals

By comparing combined data (collapsed across the vehicle and the two BN doses) from restrained animals with combined data from the unrestrained animals, we found that restraint resulted in lowered metabolic activities in LD, which includes LDDM and LDVL.

Anatomically, LD lies just caudolateral to the anterior thalamus, and peripheral to the internal medullary lamina (Faull and Mehler, 1985). Although LD is not considered a core component of the limbic circuitry, it has projections to the limbic regions (Kelly and Dodd, 1991), and the posterior cortical areas (Faull and Mehler, 1985). Functionally, it participates in the mediation of emotional expression in the mammals (Kelly and Dodd, 1991).

Emotional experiences are functions of the cingulate cortex, the temporal lobe, and the orbitofrontal cortex (Kupfermann, 1991b), whereas the behavioral expression of the emotion needs the participation of

subcortical limbic structures (Isaacson, 1974). Our observation that behavioral restraint decreased metabolic rate in LD, supports the suggestion that the mediation of animal's expression of emotions is participated in by LD, and that the animal's emotional state can be reflected in animal behaviors.

Physical restraint may reduce neural activities in the visual pathways

Similarly, by comparing combined data from restrained animals with that of the unrestrained animals, we found that behavioral restraint is associated with a decrease in LCGU in LG. In comparison, an earlier study showed that complete restraint increased LCGU in LG, although other subcortical visual structures were not affected (Bryan Jr *et al*, 1983). The discrepancy could result from the difference in restraining techniques. In the earlier study, the restraint was achieved using a neuromuscular blocker in awake animals. In our study, the animals were restrained using a jacket which is believed to be less stressful. It is suggested that the form of immobilization in our study may have reduced visual processing requirements in the animals.

Notes on methodology

An assumption of the operational equation of the [^{14}C]2DG method is that arterial plasma glucose concentration remains stable during the experimental period (Savaki *et al*, 1980; Sokoloff *et al*, 1989). Due to BN-induced hyperglycemia, the original operational equation is not applicable to ICV BN studies. We therefore calculated LCGU using the modified operational equation (Appendix 2.) (Savaki *et al*, 1980) which compensates for the effects of blood glucose fluctuation.

The classic quantitative [^{14}C]2DG method uses partial restraint to

facilitate blood sampling, reduce catheter dead space, and minimize the risk of damage to the catheters by the animals (Sokoloff *et al*, 1986; Crane and Porrino, 1989). Partially restrained animals show signs of stress, including vocalization, excessive elimination, and struggling. The plasma glucose levels are also higher than in freely-moving animals (Crane and Porrino, 1985).

We did not use partial restraint. Instead, complete physical restraint was used. Previously reported LCGU effects of complete restraint in awake, paralyzed, and artificially ventilated animals (Bryan Jr *et al*, 1983) can be compared with our data. It is believed that the stress induced by a neuromuscular blocker in an awake animal is severe, as artificial ventilation through an intratracheal intubation is very painful, especially when the restraint is maintained over a 45 min period. Restraint by a cloth jacket can hardly be considered comfortable. One of the signs of stress is that plasma glucose decrease after ICV injection in unrestrained vehicle animals (due to food deprivation during the experiment) was compensated by the restraint or BN central administration (Table 13; Figure 4).

Conclusions

Central bombesin (BN) administration induces a syndrome of stereotypic grooming and locomotion in rats. BN receptor mapping studies have provided clues as to the location of BN's central sites of action. We used the [^{14}C]deoxyglucose method to map regional functional activity following BN intracisternal injection, in both freely moving and restrained rats. Glucose utilization levels increased in the anteroventral thalamic nucleus, especially its ventral lateral portion, suprachiasmatic nucleus, internal granular layer of the olfactory bulb, and median eminence. These effects were present under both restraint conditions, and did not result from BN-induced behavioral stereotypy. Therefore, it is suggested that the metabolic alterations result from functional alterations BN receptors, and from the actions of BN-containing neurons upon their terminal projection fields. In particular, projections of BN receptor containing neurons affect cholinergic synaptic activity in the anterior thalamus.

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Appendices:

I. Key for Abbreviations

Abbreviations are in alphabetic order.

Cerebral structures:

3	oculomotor nucleus (bulbular)
Acb	accumbens nucleus (limbic)
AD	anterodorsal thalamic nucleus (thalamic)
AM	anteromedial thalamic nucleus (thalamic)
Arc	arcuate hypothalamic nucleus (hypothalamic)
AV	anteroventral thalamic nucleus (thalamic)
AVVL	anteroventral thalamic nucleus, ventral lateral (thalamic)
BLA	basolateral amygdaloid nucleus, anterior part (limbic)
Cg1	cingulate cortex, area 1 (limbic)
Cg2	cingulate cortex, area 2 (limbic)
Cg3	cingulate cortex, area 3 (limbic)
DG	dentate gyrus (limbic)
DM	dorsal medial hypothalamic nucleus (hypothalamic)
Ent	entorhinal cortex (limbic)
Hi	hippocampus (limbic)
IGr	internal granular layer of the olfactory bulb (sensory: olfactory)
InfS	infundibular stem (hypothalamic)
La	lateral amygdaloid nucleus (limbic)
LC	locus ceruleus (bulbular: reticular)
LDDM	lateral dorsal thalamic nucleus, dorsal medial (thalamic)
LDVL	lateral dorsal thalamic nucleus, ventral lateral (thalamic)
LG	lateral geniculate (thalamic)
LH	lateral hypothalamic area (hypothalamic)
LM	lateral mammillary nucleus (hypothalamic)
MD	mediaodorsal thalamic nucleus (thalamic)
ME	median eminence (hypothalamic)
MeA	medial amygdaloid nucleus (limbic)
Mi	mitral cell layer of the olfactory bulb (sensory: olfactory)
MM	medial mammillary nucleus, medial part (hypothalamic)
NcDL	deep layer of neocortex (cortical)
Pe	periventricular hypothalamic nucleus (hypothalamic)
PH	posterior hypothalamic area (hypothalamic)
PMCo	posteromedial cortical amygdaloid nucleus (limbic)
S	subiculum (limbic)
SC	superior colliculus (limbic)
SCh	suprachiasmatic nucleus (hypothalamic)
ST	stria termilanis nucleus (limbic)
Sol	nucleus of the solitary tract (bulbular: reticular)
SubGel	substantia gelitinosa (spinal)
Tu	olfactory tubercle (sensory: olfactory)
VL	ventral lateral thalamic nucleus (thalamic)
VM	ventromedial thalamic nucleus (thalamic)
VMH	ventromedial hypothalamic nucleus (hypothalamic)
VPL	ventral posterolateral thalamic nucleus (thalamic)
VPM	ventral postomedial thalamic nucleus (thalamic)

Peptides:

^{125}I-GRP	^{125}I-labeled gastrin-releasing peptide
ACTH	adrenocorticotropic hormone
$ACTH_{1-24}$	adrenocorticotropic hormone 1-24*
αMSH	α-melanocyte-stimulating hormone
BN	bombesin
BN_{6-3}	bombesin 6-3; BN specific antagonist
CR-1409	specific CCK antagonist
GH	growth hormone
GRP	gastrin releasing peptides
GRP_{1-16}	gastrin releasing peptides 1-16, GRP antagonist
GRP_{14-27}	gastrin releasing peptides 14-27
MBNs	mammalian bombesin-like peptides
NMB	neuromedin B
NMB_{30}	neuromedin B 30
NMB_{32}	neuromedin B 32
NMC	neuromedin C
SP	substance P
TRH	thyrotropin releasing hormone
TSH	thyroid stimulating hormone
ψBN**	[Leu^{13} ψ(CH_2NH) Leu^{14}]BN
[^{125}I-Tyr^4] BN*	iodine 125 labeled tyrosine BN
[^{125}I-Tyr^4] NMB	iodine 125 labeled tyrosine NMB
[D-Arg^1 D-$Trp^{7,9}$ Leu^{11}]SP	spantide BN antagonist

* Numbers refer to the amino acid position from the N-terminus;

** the ψ symbol indicates substitution of a reduced peptide bond at the indicated site *i.e.* CONH changes to CH_2NH

Biochemical substances:

2DG	2-deoxyglucose
5-HT	5-hydroxytryptamine
6-OHDA	6-hydroxydopamine
AChE	acetylcholinesterase
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
DG-6-P	2-deoxyglucose-6-phosphate
G-6-P	glucose-6-phosphate
GABA	γ-aminobutyric acid
LCGU	local cerebral glucose utilization
[^{14}C]2DG	[^{14}C]deoxy-D-glucose
[^{14}C]DG-6-P	[^{14}C]deoxy-glucose-6-phosphate

Abbreviations for Amino Acids:

Ala	alanine
Arg	arginine
Asn	asparagine
Cpa	*p*-chlorophenylalanine
Gln	glutamine
Glu	glutamic acid
Gly	glycine
His	histamine
Leu	leucine
Met	methionine
pGlu	pyroglutamic acid
Phe	phenylalanine
Pro	proline
Thr	threonine
Trp	tryptophan
Tyr	tyrosine
Val	valine

Other:

ICV	intracerebraoventricular
LCGU	local cerebral glucose utilization

II. Equations used in LCGU calculation

General Equation for Measurement of Reaction Rates with Tracers:

$$\text{Rate of Reaction} = \frac{\text{Labeled Product Formed in Interval of Time, 0 to T}}{\left[\begin{array}{c}\text{Isotope Effect}\\ \text{Correction Factor}\end{array}\right]\left[\begin{array}{c}\text{Integrated Specific Activity}\\ \text{of Precursor}\end{array}\right]}$$

Operation Equation of [^{14}C]Deoxyglucose Method:

$$R_i = \frac{\overbrace{\overbrace{C_i^*(T)}^{\text{Total } {}^{14}\text{C in Tissue at time, T}} - \overbrace{K_1^* e^{-(k_2^*+k_3^*)T}\int_0^T C_p\, e^{(k_2^*+k_3^*)t}\,dt}^{{}^{14}\text{C in Precursor Remaining in Tissue at Time, T}}}^{\text{Labeled Product Formed in interval of Time, 0 to T}}}{\underbrace{\left[\frac{\lambda V_m^* K_m}{\Phi V_m K_m^*}\right]}_{\text{Isotope Effect Correction Factor}}\underbrace{\left[\int_0^T\left(\frac{C_p^*}{C_p}\right)dt - e^{-(k_2^*+k_3^*)T}\int_0^T\left(\frac{C_p^*}{C_p}\right)e^{(k_2^*+k_3^*)t}\,dt\right]}_{\text{Integrated Plasma Specific Activity}\quad\text{Correction for Lag in Tissue Equilibration with Plasma}}}$$

Integrated Precursor Specific Activity in Tissue

Modified Operational Equation:

$$R_i = \frac{C_i^*(t) - k_1^* \exp-(k_2^*+k_3^*)t\int_0^t C_p^* \exp(k_2^*+k_3^*)t\,dt}{\frac{\lambda V_m^* K_m}{\Phi V_m K_m^*}\int_0^t\left[\frac{(k_2^*+k_3^*)\exp-(k_2^*+k_3^*)T\int_0^T C_p^*\exp(k_2^*+k_3^*)t\,dt}{C_p(0)\exp-(k_2+k_3)T + (k_2+k_3)\exp-(k_2+k_3)T\int_0^T C_p \exp(k_2+k_3)t\,dt}\right]dT}$$

III. LCGU for the structures used in this study

Local cerebral glucose utilization (LCGU, µmol/100g/min): Primary structures (Mean±S.E.; n=8).

Structures	Vehicle Unrestr.	BN 0.1µg Unrestr.	BN 0.5µg Unrestr.	Vehicle Restr.	BN 0.1µg Restr.	BN 0.5µg Restr.
AD	66.45 ±1.72	78.50 ±8.64	68.87 ±3.67	53.42 ±3.03	66.45 ±7.80	71.55 ±4.79
AM	59.87 ±2.78	62.11 ±5.35	54.46 ±1.27	54.66 ±4.01	62.30 ±8.76	63.46 ±5.10
AV	69.68 ±2.87	85.23 ±9.27	71.65 ±3.64	53.79 ±3.32	70.51 ±8.50	76.69 ±5.61
AVVL	73.45 ±3.58	108.00 ±12.22	116.61 ±4.51	61.26 ±3.94	100.36 ±13.26	106.83 ±8.15

Local cerebral glucose utilization (LCGU, µmol/100g/min): Secondary structures (Mean±S.E.; n=8).

Structures	Vehicle Unrestr.	BN 0.1µg Unrestr.	BN 0.5µg Unrestr.	Vehicle Restr.	BN 0.1µg Restr.	BN 0.5µg Restr.
IGr	61.38 ±4.31	74.59 ±10.77	63.36 ±4.94	61.65 ±3.13	88.75 ±11.63	74.18 ±8.49
Mi	61.85 ±5.83	69.20 ±10.05	61.70 ±5.82	57.97 ±3.33	84.24 ±10.79	68.50 ±7.46
Tu	55.91 ±4.52	65.24 ±4.48	56.43 ±4.01	59.52 ±5.36	52.92 ±6.50	60.41 ±3.51
NcDL	50.37 ±4.55	44.98 ±4.91	41.30 ±3.72	45.38 ±4.06	43.59 ±5.63	43.78 ±3.66
Acb	46.65 ±4.75	49.16 ±3.35	43.48 ±2.34	47.59 ±3.93	45.06 ±5.93	47.89 ±3.34
DG	32.16 ±3.41	34.44 ±3.39	29.33 ±2.26	29.06 ±1.71	33.01 ±2.59	30.38 ±1.59
S	50.19 ±3.81	49.91 ±4.57	40.58 ±2.55	41.77 ±3.43	42.86 ±4.03	42.97 ±3.49
ST	27.86 ±3.19	31.80 ±3.51	31.38 ±2.09	25.78 ±1.68	26.31 ±2.96	28.27 ±2.69
BLA	51.53 ±3.87	46.15 ±4.13	47.22 ±2.42	46.37 ±2.93	48.39 ±6.46	48.73 ±2.98
La	35.65 ±2.89	33.67 ±3.67	33.91 ±2.14	35.14 ±1.77	34.51 ±5.05	34.37 ±2.46
MeA	33.00 ±2.91	33.15 ±2.22	34.80 ±2.94	34.40 ±2.37	38.55 ±4.98	34.71 ±3.00
PMCo	38.86 ±3.88	42.80 ±1.93	34.80 ±2.72	38.75 ±2.61	45.41 ±4.43	43.66 ±2.94
MD	60.94 ±4.31	59.95 ±6.02	58.01 ±3.44	54.28 ±2.82	61.16 ±7.74	59.50 ±4.80
Pe	28.52 ±2.75	28.89 ±2.54	26.97 ±1.29	30.64 ±1.62	28.77 ±2.75	29.73 ±2.39
SCh	33.47 ±1.65	40.66 ±3.89	47.32 ±4.84	34.45 ±2.37	39.90 ±5.25	50.96 ±5.24
LC	47.75 ±5.22	41.68 ±2.86	43.75 ±2.22	43.04 ±3.31	46.93 ±5.93	43.63 ±1.94
Sol	41.97 ±2.72	35.44 ±2.91	34.8 ±2.65	36.88 ±2.51	41.71 ±6.00	35.06 ±7.67
SubGel	41.17 ±3.94	49.98 ±5.72	43.83 ±5.92	39.37 ±3.57	55.87 ±6.92	58.94 ±6.94

Local cerebral glucose utilization (LCGU, µmol/100g/min)
Tertiary structures (Mean±S.E.; n=8.)

Structures	Vehicle Unrestr.	BN 0.1µg Unrestr.	BN 0.5µg Unrestr.	Vehicle Restr.	BN 0.1µg Restr.	BN 0.5µg Restr.
Cg1	59.96 ±6.31	55.22 ±5.18	51.73 ±4.02	56.51 ±4.97	53.73 ±6.52	58.18 ±3.98
Cg2	55.59 ±5.27	54.46 ±5.64	50.26 ±3.39	55.37 ±4.94	49.46 ±5.83	54.31 ±3.66
Cg3	52.64 ±5.67	53.07 ±4.47	48.19 ±4.09	53.66 ±4.15	55.5 ±7.89	52.83 ±3.93
Hi	41.19 ±3.00	39.44 ±2.09	34.02 ±1.82	38.34 ±2.26	41.59 ±5.97	38.99 ±2.51
Ent	41.49 ±3.44	43.16 ±3.56	35.05 ±1.84	38.49 ±2.38	44.83 ±5.22	43.81 ±3.35
LDDM	60.26 ±4.31	60.48 ±4.77	62.67 ±2.80	50.7 ±2.81	54.68 ±7.78	54.00 ±4.00
LDVL	57.64 ±4.11	59.20 ±4.69	63.7 ±4.40	45.40 ±1.99	53.26 ±7.88	50.01 ±3.42
VL	62.02 ±4.38	60.00 ±4.45	62.45 ±2.57	53.02 ±2.51	55.31 ±6.45	57.94 ±4.36
VM	68.08 ±3.76	65.98 ±5.31	64.85 ±3.18	58.54 ±3.32	63.19 ±8.42	62.85 ±3.93
VPL	58.12 ±4.61	54.12 ±4.30	59.72 ±3.26	48.14 ±2.54	50.49 ±7.34	49.85 ±3.93
VPM	57.88 ±5.44	55.98 ±3.31	59.31 ±5.42	49.79 ±3.35	51.62 ±6.38	48.45 ±3.03
LG	62.65 ±3.93	68.42 ±4.15	58.49 ±3.99	49.19 ±2.60	55.50 ±6.41	60.33 ±4.26
VMH	31.94 ±2.93	29.50 ±2.16	32.59 ±2.90	32.97 ±1.62	37.23 ±5.23	34.99 ±2.89
DM	36.89 ±3.07	33.07 ±1.73	30.96 ±2.59	35.48 ±1.40	37.25 ±2.73	33.20 ±3.07
LH	37.93 ±3.11	39.23 ±3.55	38.03 ±2.60	40.47 ±3.89	40.87 ±4.84	36.38 ±2.49
PH	39.38 ±4.24	39.69 ±3.10	31.76 ±3.46	38.97 ±2.79	42.12 ±5.19	39.03 ±3.74

(continued)

(continue)

Structures	Vehicle Unrestr.	BN 0.1µg Unrestr.	BN 0.5µg Unrestr.	Vehicle Restr.	BN 0.1µg Restr.	BN 0.5µg Restr.
Arc	28.89 ±2.58	30.89 ±1.98	36.81 ±4.00	33.21 ±2.03	38.79 ±4.24	35.04 ±3.58
InfS	29.30 ±3.62	38.93 ±2.52	42.38 ±4.05	38.35 ±3.87	40.25 ±7.15	46.46 ±4.88
ME	35.39 ±2.32	45.32 ±5.16	59.12 ±5.96	41.49 ±5.66	47.50 ±8.20	56.63 ±7.83
MM	80.77 ±5.00	90.18 ±7.59	75.67 ±4.42	64.24 ±3.55	86.41 ±11.60	83.90 ±6.66
LM	79.24 ±5.92	88.43 ±7.58	77.40 ±5.25	56.17 ±3.03	84.09 ±11.04	76.15 ±6.73
3	67.03 ±4.36	66.91 ±7.06	54.95 ±3.77	53.37 ±4.40	60.09 ±6.12	61.71 ±4.74
SC	57.09 ±3.03	58.38 ±4.34	45.85 ±3.58	48.19 ±3.65	52.68 ±5.17	52.49 ±4.87